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The Mechanism of Ski's Repressive Effects on Retinoic Acid Signaling Pathway

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Abstract of the Dissertation

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C-Ski is the founding member of a family of proteins that includes itself and Ski-related protein Sno and it is a physiologically relevant negative regulator of signaling by Retinoic Acid (RA), which is essential for mammalian development. Tight regulation of RA regulated signaling pathway is essential for physiological responses. The mechanism by which Ski represses RA signaling is unknown. In this study, we examined how c-Ski could inhibit RA-induced transcriptional activation. Co-immunoprecipitation and immunofluorescence assay showed that Ski and RARα were in the same complex in both the absence and presence of RA, which made Ski different from other co-repressors since the components of co-repressor complex for RA signaling undergo proteasome

degradation upon RA treatment. We determined that Ski could stabilize overexpressed RARα and HDAC3, a key functional member of the co-repressor complex, even in the presence of RA. We further demonstrated that the endogenous levels of HDAC3 could also be stabilized by the regulated Ski expression. Using shRNA that directed against Ski to reduce the levels of endogenous Ski protein in MG63 osteosarcoma cell line, we found that reduction in the level of expression of Ski led to a parallel reduction of HDAC3 levels. Together these data suggest that Ski represses RA signaling by stabilizing components of the co-repressor complex. Furthermore, we showed that TBLR1, an F box protein, could target HDAC3 for proteasome degradation, which was partially abrogated by the presence of Ski. Siah2, an E3 ligase, was able to interact with HDAC3 and mediate its ubiquitin-proteasome degradation. In addition, even though we did not detect interaction between RARα and Siah2, we still found enhanced RARα degradation by the expression of Siah2. Interestingly, we found the Ski protein was in a complex with Siah2. Moreover, only wild type Ski and Ski mutants that were in the same complex with Siah2 could stabilize RARα and HDAC3. The expression level of Siah2 was increased when Ski was co-expressed, which indicated the inhibitory effects of Ski on Siah2 activity. Taken together, we propose that the Ski protein inhibits RA signaling pathway through maintaining the basal repressed state of the RA target genes by inhibiting TBLR1 and/or Siah2 mediated proteasome degradation of the co-repressor complex.

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Publications

The majority of the work contained in Chapter II was originally published in the following citation:

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Chapter I

Introduction

The discovery of Ski and the Ski/Sno family

V-Ski, a truncated form of the cellular homolog c-Ski, was first identified as the oncoprotein encoded by the avian Sloan-Kettering retroviruses and was responsible for their ability to transform avian fibroblasts in vitro (Li *et al.*, 1986). H-Ski, the human Ski gene which is 91% homologous with v-Ski at the amino acid level , and the Ski-related novel gene, Sno are isolated by screening human cDNA libraries (Nomura *et al.*, 1989). In addition to human Ski, a homolog of c-Ski has been identified in chicken (Stavnezer *et al.*, 1986), xenopus (Sleeman and Laskey, 1993), mouse (Namciu *et al.*, 1995) and zebra fish (Kaufman *et al.*, 2000) but not in any lower eukaryotes.

C-Ski is the founding member of a family of proteins that includes itself and the Ski-related protein Sno. Recently, two proteins Fussel-18 (Arndt *et al.*, 2005) and Fussel-15 (Arndt *et al.*, 2007) have been cloned and characterized as two new members of the Ski family. The human Ski gene encodes a protein of 728 amino acids and has not been reported to be regulated by alternative splicing even though three alternatively spliced forms of chicken Ski have been characterized (Sutrave and Hughes, 1989). There are several isoforms of Sno proteins in human, which result from alternative splicing. The full-length Sno gene encodes a 684 amino acids protein, SnoN. The isoforms include SnoN2, which is produced by alternative splicing within exon3 of SnoN, SnoI, encoding a truncated isoform of the first 399 amino acids, and SnoA, which encodes a protein of 415 amino acid residues and differs from SnoN after the first 366 amino acid residues (Nomura *et al.*, 1989; Pearson-White, 1993; Pearson-White and Crittenden, 1997). The two new members of the Ski/Sno family, Fussel-18 (functional smad suppressing element on chromosome 18), encoded a protein of 297 amino acids and Fussel-15 (functional

smad suppressing element on chromosome 15), encoded a protein of 921 amino acids. They share characteristic structural features, significant homology and similar genomic organization with the two original Ski family members (Arndt *et al.*, 2007; Arndt *et al.*, 2005).

The members of the Ski/Sno family share significant homology between each other and contain several structural domains (Figure 1.1 Ski/Sno family and their functional domains). The Ski and SnoN proteins share an overall 50% amino acid homology, and Fussel-15 and Fussel-18 proteins show 86% homology to each other and build a subfamily to Ski/SnoN with 28% amino acid homology to this family (Arndt *et al.*, 2007). They also share similar domain structures, including R-Smad binding domain, the Dachshund homology domain (DHD) in their N-terminal region (Kozmik *et al.*, 1999), a Smad4 binding domain (Wu *et al.*, 2002), and a less conserved carboxyl terminal domain (Heyman and Stavnezer, 1994; Zheng *et al.*, 1997). The residues 17-46 were reported to be sufficient for the interaction of Ski with the phosphorylated, trimeric form of Smad 2/3 (Qin *et al.*, 2002). The DHD (residues 98-192 of Ski) forms a compact, globular domain consisting of mixed alpha helix and beta sheets, which is similar to the structure found in the forkhead/winged-helix family of DNA binding proteins (Wilson *et al.*, 2004). Although DHD of the Ski family is not a DNA binding domain since neither Ski nor SnoN has been reported to directly bind DNA, it is responsible for their interaction with other proteins. Residues 99-274 are responsible for N-CoR binding (Nomura *et al.*, 1999), and the Skip interaction is mapped to the residues 95-202 (Dahl *et al.*, 1998b; Prathapam *et al.*, 2001), both of which are in the DHD. DHD of Ski may also involved in Smad3 binding (Ueki and Hayman, 2003a) by providing the scaffold for presenting the R-Smad

domain of Ski. Adjacent to the DHD is the Smad4 binding domain of Ski, which is a C2H2 Zn-binding module with structural homology to the SAND domain (named after Sp100, AIRE1, NucP41/75, DEAF1) (Wu *et al.*, 2002). The C-terminal regions of Ski and SnoN are less conserved, but are required for Ski oligomerization. There are five tandem repeats and an α helical leucine zipper motif in this region, which are responsible for Ski homodimer formation and Ski-SnoN heterodimerization, respectively (Cohen *et al.*, 1999; Heyman and Stavnezer, 1994).

The biological function of the Ski/Sno family

The expression pattern and roles in vertebrate development

Ski has been identified in human, xenopus, mouse and zebrafish and is ubiquitously expressed in all adult tissues at low levels (Nomura *et al.*, 1989; Pearson-White and Crittenden, 1997; Wu *et al.*, 2003). It can regulate growth and differentiation of several cell types, especially those in neural and muscle lineages. It has been shown that Ski can control Schwann cell proliferation and myelination by being in the same complex with Oct6, which is a myelination-regulating transcription factor (Atanasoski *et al.*, 2004). Overexpression of Ski stimulates the proliferation, anchorage-independent growth and induces muscle differentiation of quail embryo fibroblasts (Colmenares and Stavnezer, 1989). Furthermore, in vivo studies have shown that the Ski protein plays an important role in the neuronal and skeletal muscle development (Amaravadi *et al.*, 1997; Berk *et al.*, 1997; Kaufman *et al.*, 2000).The Xenopus Ski protein (XSki) is maternal and distributed widely in the early gastrula embryo. Ectopic overexpression of the Ski leads to autonomous neural axis formation and neuroral specific gene expression in ectoderm explants (Amaravadi *et al.*, 1997). In mouse, low levels of Ski are found in all cells of the

developing mouse and relatively high levels are found at certain stages of embryonic and postnatal development. In the mouse embryo, the Ski expression increases at E8.5 to E9.5, and this corresponds to neural tube closure and neuronal crest cells migration (Lyons *et al.*, 1994). At about E12.5, elevated levels of Ski mRNA can be detected in the skeletal muscle, which correlates with terminal differentiation of skeletal muscle cells (Namciu *et al.*, 1995). At E14 to E16, high levels of Ski mRNAs are detected in the cortical layers of the brain and in the olfactory bulb. Two-fold increase of Ski is also found in the neonatal lung tissue (Lyons *et al.*, 1994). This expression pattern implies that Ski plays a role in both the proliferation and differentiation of specific cell populations of the central and peripheral nervous systems and of other tissues. This conclusion is supported by the phenotype of the Ski-null mice. Exencephaly, resulting from the failed closure of the cranial neural tube during neurulation, and a dramatic reduction in skeletal muscle mass, resulting from a defect in expansion of a myogenic precursor population, have been observed in these mice (Berk *et al.*, 1997). In mice, loss of Ski also leads to reduced skeletal muscle mass (Berk *et al.*, 1997; Colmenares *et al.*, 2002), whereas in transgenic mice Ski overexpression causes large increases in skeletal muscle, which is the result of the type II fast fibers hypertrophy (Sutrave *et al.*, 1990b). Consistent with these results, Ski has been shown to activate the expression of muscles-specific genes, myogenin and muscle creatine kinase through transcriptional activation (Kobayashi *et al.*, 2007; Mimura *et al.*, 1996). In humans, the Ski gene maps to chromosome 1p36.3. Terminal deletions of chromosome 1 are seen in humans giving rise to monosomy 1p36 syndrome (Slavotinek *et al.*, 1999). Children with this syndrome have craniofacial defects and digit defects, which are virtually identical to those seen in the $Ski^{-/-}$ mice (Colmenares *et al.*, 2002),

implying that these defects may be due to reduced levels of Ski. In addition to neural and muscle cells, Ski also plays an important role in hematopoietic cells growth and differentiation (Dahl *et al.*, 1998a; Pearson-White *et al.*, 1995).

Like Ski, SnoN expresses in most tissues and can regulate growth and differentiation of several cell types, especially those in neural and muscle lineages. Even though it can induce muscle differentiation of quail embryo fibroblast, compared to c-Ski this happens only at relatively high levels of SnoN. SnoN is also involved in the T-cell proliferation and activation since defective T-cells proliferation in response to activating stimuli has been exhibited in the Sno gene mutated mice (Pearson-White and McDuffie, 2003). The SnoN protein shares highly homologous domains with Ski in the amino terminus, but the function of SnoN in development is different from that of Ski. Until now, three lines of Sno-knockout mice have been generated and examined. First, Sno knock-out mice died at an early stage of embryogenesis and Sno was required for blastocyst formation, which implicated an important role of Sno in the early development of mouse embryos (Shinagawa *et al.*, 2000). However, mice with other two null mutations in the Sno gene were reported to be viable (Pearson-White and McDuffie, 2003). Since this discrepancy has not been fully clarified, the role of SnoN in embryonic development remains unclear.

In contrast to Ski and SnoN, which are ubiquitously expressed in tissues, the other two members of the Ski/Sno family Fussel-15 and Fussel-18 are much more restricted in expression and found specifically in the nervous system (Arndt *et al.*, 2007; Arndt *et al.*, 2005). In addition to the central nervous system, fussel-15 is also found in the developing lung and bladder (Arndt *et al.*, 2007) .

The dual function of Ski/SnoN in tumorigenesis: the pro-oncogene and the tumor suppressor

C-Ski, v-Ski and SnoN are classified as oncoproteins based on their abilities to transform avian embryo fibroblasts *in vitro* when they are overexpressed (Boyer *et al.*, 1993; Li *et al.*, 1986; Stavnezer *et al.*, 1981). Ski and SnoN have also been shown to be critical in neuronal and muscle cells differentiation (Amaravadi *et al.*, 1997; Berk *et al.*, 1997; Kaufman *et al.*, 2000) and play important roles in mouse embryo development. These data have established the importance and complexity of the function of Ski/SnoN family. We now know that Ski/SnoN can act as both pro-oncogene and tumor suppressor.

The pro-oncogenic function

The expressions of Ski and SnoN are up regulated in many tumors. In esophageal squamous cell carcinomas, both Ski and SnoN are found overexpressed (Akagi *et al.*, 2008; Fukuchi *et al.*, 2004; Imoto *et al.*, 2001), and furthermore, high levels of Ski expression correlate with the progress of this carcinoma, specifically with depth of invasion and pathologic stage (Fukuchi *et al.*, 2004). High levels of SnoN can be a sign of poor survival in patients with esophageal squamous cell carcinoma (Akagi *et al.*, 2008). Ski and SnoN expressions have also been found in human melanoma tumor tissues and cells (Poser *et al.*, 2005; Reed *et al.*, 2001) and the levels of Ski expression correlate with the malignant grade of this tumor. Furthermore, the Ski expression pattern changes from nucleus in preinvasive melanomas to nucleus and cytoplasm in invasive and metastatic melanomas (Reed *et al.*, 2001). The importance of Ski and SnoN overexpression in melanoma progress is further confirmed by knocking down their levels using antisense Ski vectors, which slows down the growth of melanoma cells (Poser *et al.*, 2005; Reed *et*

al., 2001). Ski and SnoN can also be prognostic markers in early colorectal cancer and estrogen receptor-positive breast carcinomas, respectively (Buess *et al.*, 2004; Zhang *et al.*, 2003). In addition, it has been shown that Ski can cause highly malignant erythroleukemia (Larsen *et al.*, 1992) and transform hematopoietic multipotent progenitors (Dahl *et al.*, 1998a). Increased expression of Ski can induce immortalization and self-renewal of primary multipotential myeloid progenitor cells from avian bone marrow (Beug *et al.*, 1995).

The tumor suppressor

Recently, more and more evidence suggest that Ski and SnoN play complicated roles in cancer development and may act as tumor suppressors in some types of tissues and cells. Even though high level of SnoN is found in many human cancer cell lines and is responsible for mitogenic transformation of breast and lung cancer cell lines in vitro and tumor growth in vivo, SnoN overexpression can also inhibit epithelial-to-mesenchymal transdifferentiation and thus inhibit tumor metastasis. This tumor suppressor characteristic is further supported by the observation that down-regulation of SnoN by shRNA can moderately enhance metastasis of human breast cancer cells to the bone and lung (Zhu *et al.*, 2007). Depending on the stage of tumor development, SnoN plays either tumor promotion or tumor suppression activity. High levels of Ski and SnoN are detected in Barrett's esophagus tissue, which is a precancerous condition. Markedly decreased Ski and SnoN levels are associated with tissue samples from patients with low-grade dysplasia and the absent expression of Ski and SnoN are found in those patients with high-grade dysplasia or adenocarcinoma (Villanacci *et al.*, 2008). Furthermore, heterozygous $sno^{+/-}$ mice are more sensitive to a chemical carcinogen compared to wild

type mice and show spontaneous lymphomas and increased levels of tumor formation (Shinagawa *et al.*, 2000). Similar to SnoN, Ski can also act as a tumor suppressor under certain condition. First, ski $^{+/-}$ mice also show increased susceptibility to tumorigenesis when they are challenged with a chemical carcinogen (Shinagawa *et al.*, 2001), which suggests that the extra copy of the Sno and Ski gene can actually protect mice against carcinogenesis and they may play roles as tumor suppressors. Then researchers reported that reducing Ski expression in breast and lung cancer cells enhances tumor metastasis in vivo without affecting tumor growth (Le Scolan *et al.*, 2008). Finally, Ski can repress the oncogenic activation of c- Myb, a transcription factor associated with myeloid leukemia, which could be one mechanism by which Ski acts as a tumor suppressor (Nomura *et al.*, 2004).

Ski and SnoN seem to live a dual life, one as an oncogene and the other as a tumor suppressor. Until now, there is no general mechanism, which can explain the complex functions of these two proteins since they are involved in lots of signaling pathways and play functions from embryonic development to tumorigenesis. There might be a unique mechanism for every event. Depending on the genetic background of different tissues and cells, the stage of development and tumorigenesis, the binding partner of Ski and SnoN might undergo changes, and even different expression levels of Ski and SnoN could lead to changes of their interaction partners from ones that they normally bind to those they do not normally bind.

Ski/SnoN family and TGF-β superfamily

The members of Ski/SnoN family are important negative regulators for TGF-β superfamily, which includes TGF-β (Transforming Growth Factor- β), BMPs (Bone

Morphogenic Proteins), nodals, activins and anti-Müllerian hormone (Massague, 1998; Massaous and Hata, 1997). TGF-β family plays important roles in variety of biological processes, such as vertebrate development, immune responses, cell growth, differentiation, and apoptosis (Sporn and Roberts, 1990). Since Ski/SnoN family is a repressor of signaling by the TGF-β family, they are also involved in these processes (Luo, 2004).

TGF-β, which is a member of disulfide-bonded cytokines, is a potent growth inhibitor for most types of cells. Active TGF-β can bind to type II and type I serine/threonine kinase receptors on the cell membrane and activate type II receptor, then the activated type II receptor transphosphorylate and activate type I receptor kinase. Once the type I receptor kinase is activated, TGF-β initiates diverse cellular response through various intracellular signaling pathways. Smad proteins are responsible for the transduction of TGF-β signaling from cytoplasm to nucleus (Heldin *et al.*, 1997). There are three subtypes of Smads proteins, receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads) (Massaous and Hata, 1997). In TGF-β signaling pathway, Smad2 and Smad3 are the R-Smads, Smad4 acts as a Co-Smad and Smad7 is the I-Smad. Smad2 and Smad3 are anchored to the plasma membrane through Smad Anchor for Receptor Activation (SARA) (Tsukazaki *et al.*, 1998) where they are phosphorylated by activated type I receptor kinase, the phosphorylated R-Smads, Smad2 and Smad3, form a heteromeric complex with Co-Smad, Smad4 and translocate into the nucleus. Here the Smad2/3-Smad4 heteromers interact with various transcription factors, transcriptional co-activators, or co-repressors, which lead to transcriptional activation or repression of target genes in the target cells

(Miyazono *et al.*, 2000). Ski and SnoN can negatively regulate TGF-β signaling by binding to the Smads proteins (Luo, 2004), which may happen in both the cytoplasm and the nucleus. There are several possible mechanisms involved in this repression. First, Ski might interrupt the Smad2/3-Smad4 heteromer formation by competing with phosphorylated Smad2 for Smad4 binding (Wu *et al.*, 2002). Second, Smad2/3-Smad4 heteromeric complexes still form, but they are inactive and stabilized on the promoter region of the TGF-β target genes to prevent further binding of newly synthesized and active Smads complex (Suzuki *et al.*, 2004). Third, high expression of Ski and SnoN may inhibit the binding of Smads complex to transcriptional co-activator p300/CBP (Akiyoshi *et al.*, 1999; Wu *et al.*, 2002) and recruit transcriptional repressor complex including N-CoR /SMRT and HDAC to the promoter region of target genes (Luo *et al.*, 1999; Stroschein *et al.*, 1999). Even though Ski and SnoN are primarily nuclear proteins and their inhibitory activities on TGF-β signaling pathway happen in nucleus, Ski expression is found in both nucleus and cytoplasm in primary invasive and metastatic melanomas, and furthermore, Ski in the cytoplasm is associated with Smad3 and prevents Smad3 from translocating into the nucleus upon TGF-β treatment (Reed *et al.*, 2001). Similar to Ski, SnoN has also been reported to be cytoplasmic in normal tissues and non-tumorigenic or primary epithelial cells (Krakowski *et al.*, 2005). Cytoplasmic SnoN inhibits TGF-β signaling through sequestering the Smad proteins and blocking the translocation of R-Smads into nucleus. In addition, it has been reported that high level of Ski might interfere with the phosphorylation of Smad2 by activated type I receptor kinase (Prunier *et al.*, 2003), which happens on the cell membrane. From these observations, we can conclude that the inhibitory effect of Ski and SnoN on TGF-β signaling pathway is

not only attributable to transcriptional repression in the nucleus but may also involve cytoplasmic inhibition, which can be either direct or indirect (Figure 1.2 Ski/SnoN and TGF-β pathway).

BMP signaling pathway regulates variety of processes during vertebrate development and is required for mesoderm formation, neurogenesis, and patterning of many different organ systems (Hogan, 1996). BMP signaling pathway also use type II and type I transmembrane Serine/Threonine receptors kinase, but the R-Smads and I-Smad are different from those in TGF-β signaling, Smads1, 5 and 8 are R-Smads and activated by BMP receptors. Smad6 is the I-Smad and can mediate BMP signaling inhibition. Upon phosphorylation, BMP specific Smads form a complex with the Co-Smad, Smad4, and translocate into the nucleus to mediate target gene transcription (Heldin *et al.*, 1997; Kusanagi *et al.*, 2000; Massague and Chen, 2000). Ski interacts with BMP-specific Smads, Smad1/5, and inhibits BMP target gene transcription (Takeda *et al.*, 2004; Wang *et al.*, 2000).

Fussel-18 and Fussel-15 are two other members in the Ski/SnoN family. Like Ski and SnoN, Fussel-18 binds to Smad2 and Smad3 and inhibits TGF-β signaling (Arndt *et al.*, 2005). Even though highly homologous to Fussel-18, Fussel-15 interacts with Smad1, Smad2 and Smad3 and represses primarily BMP signaling pathway (Arndt *et al.*, 2007).

It is well known that Ski and SnoN are two important negative regulators for TGF-β signaling pathway. Surprisingly, under certain conditions, SnoN can function as a cell type specific co-activator of TGF-β signaling in a mink lung epithelial cell line, which only happens with very low SnoN level (Sarker *et al.*, 2005). Although the

biological relevance of these observations is still elusive, it suggests complicated functions of the Ski/SnoN family.

The Ski protein and nuclear hormone receptor signaling pathway

Classification of nuclear hormone receptors

Nuclear hormone receptors are an evolutionary conserved class of ligand-activated intracellular transcription factors, which have multiple important functions in many cells and tissue types including proliferation, differentiation, apoptosis, reproduction, and metabolic homeostasis (Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995; McKenna and O'Malley, 2002). There are different ways to classify nuclear receptors. According to the type of hormones they bind to, nuclear receptors can be divided into three types. The first type is the steroids receptors, including glucocorticoid receptor (GR), progesterone receptor (PR), mineralocorticoid receptor, androgen receptor (AR), and estrogen receptor (ER). The second type is the receptors for steroid derivates, including vitamin D receptor (VDR) and non-steroids receptors including thyroid hormone receptor (TR), retinoic acid receptor (RAR), and peroxisome proliferator activated receptor (PPAR). The third type is orphan receptors. The ligands for some of these receptors have been found recently. Now ligands for some of the orphan receptors have been identified. These ligands have important roles in a variety of metabolic processes, including lipid homeostasis (Chawla *et al.*, 2001). According to the mode that they bind to their DNA response element, four classes can be found (Mangelsdorf *et al.*, 1995; Olefsky, 2001). (Figure 1.3A Classification of nuclear receptors) (Class 1) Glucocorticoid receptor (GR), progesterone receptor (PR), estrogen receptor (ER), and androgen receptor (AR) are included in the first class. Usually, these receptors are

cytoplasmic and associated with heat shock proteins, which act as molecular chaperones to maintain the unliganded receptor in a conformation ready for ligand binding. Once the ligand binds to these receptors, it can induce the conformational changes of these receptors and lead to the dissociation of the receptors from chaperone proteins. The activated receptors then translocate into the nucleus and bind to specific palindromic response elements at upstream promoter sites as homodimers. AGGTCA is a consensus sequence recognized by ER, and AGAACA is the consensus sequence for other steroid receptors. Once the activated receptors bind to DNA, they can recruit co-activator proteins and initiate transcriptional activation of target genes. (Class 2) The second class functions as heterodimers with the retinoic X receptor (RXR). They are nuclear and recognize a direct repeat response element, which is a hexanucleotide consensus sequence of AGGTCA, in the absence of ligand. For example, thyroid hormone receptor (TR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR) and vitamin D receptor (VDR) fall into this class. In the absence of ligand, the heterodimers recruit co-repressors to inhibit target genes activation. Binding of ligand in the nucleus leads to a conformational change of nuclear receptor, co-repressors dissociation, co-activators recruitment, and transcriptional activation. (Class 3 & 4) Orphan receptors that bind DNA as monomeric forms are the third class and as dimers with RXR are the fourth class.

The structure of nuclear receptors and related functions

Nuclear receptor proteins contain several modular subunits. Transcriptional activation function domain (AF-1) is in the N-terminal region of the receptors and shows weak conservation across the nuclear receptor superfamily. The other domains include a

highly conserved centrally located DNA-binding domain (DBD), a moderately conserved C-terminal ligand-binding domain (LBD), and a hinge which connects the DBD and the LBD (Bain *et al.*, 2007; Birnbaumer *et al.*, 1983; Wrange *et al.*, 1984) (Figure 1.3B The structure domains of nuclear receptors).

AF-1 can function as a ligand-independent transcriptional activator. Little is known about the structural and folding properties of this region and the sequences N-terminal to the DBD. According to the various studies on the N-terminal regions of steroid receptor, this domain tends to be unstructured or minimal folded in isolation and can acquire significant secondary and tertiary structure by exposure to interacting partners or linking to DBD (Bain *et al.*, 2007; Birnbaumer *et al.*, 1983; Wrange *et al.*, 1984). For example, the function and structure of the isolated AF-1 of GR and AR can be restored by the presence of the naturally occurring osmolyte trimethylamine N-oxide (TMAO). In the presence of TMAO, AF-1 of GR folds into a more compact structure, which is important for its interaction with certain co-regulators (Kumar *et al.*, 2001). As for AR, in the presence of TMAO, binding to the RAP74 subunit of general transcription factor TFIIF also results in a stable conformation (Reid *et al.*, 2002). DBD is another factor that can induce stable secondary or tertiary structure formation. It has been reported that the interdomain signaling is also important for the transactivation function of AF-1 domain in GR. When the AF-1 domain is linked to its DBD, additional secondary as well as tertiary structure is acquired in this two-domain proteins upon DNA binding (Kumar *et al.*, 1999). Similar situations are also observed in PR (Bain *et al.*, 2000; Bain *et al.*, 2001). The importance of partner protein interaction for the structural formation of nuclear receptor N-terminal region is further supported by the research on

ER. The N-terminal regions of ERα and ERβ are unstructured in vitro, TATA-binding protein (TBP) interaction leads to structural changes of N-terminal region of ERα. Neither interaction between N-terminal region of ERβ and TBP nor structural change of ERβ N-terminal region in the presence of TBP has been detected (Warnmark *et al.*, 2001). More interestingly, the types of structural changes might be specific to the type of promoter sequences that ER bound, which influences the recruitment of specific co-activator proteins (Wood *et al.*, 2001).

DBD, which is responsible for docking the receptor to the hexanucleotide response elements located within nuclear receptor-regulated promoters, is the most highly conserved domain among the nuclear receptors. All nuclear receptor DBD contain two α helices, the N-terminal helix forms the crucial substructure for direct interaction with the major groove of each DNA half-site, whereas the C-terminal helix packs together with helix 1 in a perpendicular fashion via their hydrophobic faces and contributes to stabilization of the overall protein structure. The DBD globular domain structure and DNA–binding activity are also maintained by two zinc-finger motifs, each zinc atom is coordinated by four cysteine residues in a tetrahedral arrangement (Freedman *et al.*, 1988).

The LBD, typically about 250 amino acids in length, includes a ligand-regulated transcriptional activation function-2 domain (AF-2) for co-activator recruitment, an interior binding pocket required for the ligand binding, and dimerization motifs (Wurtz *et al.*, 1996) (Bain *et al.*, 2007). In contrast to the weakly conserved AF-1 domain, AF-2 domain is moderately conserved across the nuclear receptor superfamily and shows a globular domain made up of 11-13 α-helices that are organized into an α-helical

sandwich which is composed of a three-layer anti-parallel helical sheet. The two outer layers of the sandwich consist of three long helices (helices 3, 7, and 10), and the middle layer, which comprises helices 4, 5, 8, and 9, presents only in the top half of the domain and is absent from the bottom half, thus creating a interior cavity for ligand binding. Furthermore, the structure of this top half of the domain is highly conserved among the LBD of various nuclear receptors, indicating evolutionarily conserved function of this sandwich fold for small molecules binding (Li *et al.*, 2003). The AF-2 domain also forms an α helix (helix 12) and is part of the hydrophobic groove, which is responsible for recruiting proteins such as the steroid receptor co-activator (SRC) family and can adopt different conformations depending on the nature of the bound ligand. In the absence of ligand, the hydrophobic groove is either in an inactive form (Johnson *et al.*, 2000) or incomplete since the AF-2 domain (helix 12) is pointing out of the core structure of the LBD, which result in an inactive LBD and no co-activators recruitment (Bourguet *et al.*, 1995). Once an activating ligand binds to the LBD, AF-2 domain (helix 12) is stabilized and forms the hydrophobic groove with the core structure of LBD (Shiau *et al.*, 1998). Co-activators contain helical LXXLL motifs, which display as a two-turn α helix, are responsible for binding via hydrophobic interactions in the groove and the subsequent chromatin-remodeling proteins and the general transcriptional activation machinery recruitment (Xu and Li, 2003). Antagonists can bind at the same site as active ligands within the core structure of LBD. However, they demonstrate different binding modes and inhibit co-activators recruitments by blocking the formation of the hydrophobic groove through preventing the AF-2 from approaching the core structure of LBD (Brzozowski *et al.*, 1997; Xu *et al.*, 2002), or by inducing AF-2 itself to mimic the

coactivators and bind in the groove unproductively (Shiau *et al.*, 1998). The conformation of AF-2 helix also determines the co-repressors recruitments such as N-CoR/SMRT. There is a conserved LXXXIXXXL/I motif amongst the nuclear receptor co-repressors, which plays an important role in the LBD binding. This motif adopts a three-turn α helix instead of two turns for the co-activator motif, and even though it binds to the same overlapped site as the LXXLL helix of the co-activators, the extra turn of LXXXIXXXL/I motif makes the co- repressors totally different from the co-activators. In the presence of an antagonist, the AF-2 helix cannot approach the core structure of LBD, which results in a larger pocket that can accommodate the larger tree-turn α-helix of the co-repressor motif. In addition, the extra turn of the co-repressor LXXXIXXXL/I motif can further prevent the AF-2 helix from acquiring its active formation by extending into the space that would normally be occupied by the AF-2 helix in its active conformation (Bain *et al.*, 2007; Li *et al.*, 2003).

The LBD and DBD connect to each other via a short amino acid sequence termed the hinge. The complete functions of this part are still elusive. Some evidence shows that the phosphorylation on the hinge is important for the efficient transactivation for some nuclear receptors (Knotts *et al.*, 2001; Lee *et al.*, 2006)

Co-regulators of nuclear receptor signaling

Nuclear receptor co-regulators, including co-activators and co-repressors, are partners of nuclear receptors and required for receptor-dependent transcriptional regulation.

Co-activators are responsible for nuclear receptor-dependent gene activation through a variety of mechanisms in the transcriptional activation process (Onate *et al.*,

1995). The co-activators include the p160 family, CREB-binding protein (CBP)/p300, p/CAF, thyroid hormone receptor (TR)-associated protein (TRAP)/vitamin D3 receptor (VDR)-interacting protein (DRIP), activating signal cointegrator-1 (ASC-1), activating signal cointegrator-2 (ASC-2), TIF1,ARA70, SRA, PGC-1, Smad3, REA, RIP140, and others (Lee *et al.*, 2001). There are several major functions for co-activators. (1) Co-activators bind to nuclear receptors or transcriptional factors in a ligand-dependent manner. (2) Some of co-activators have a chromatin-modifying enzymatic activity, which is responsible for histone modification and chromatin remodeling. (3) Some of them act as scaffold proteins, which provide a platform for recruiting and/or stabilizing the chromatin modifying co-activator complex or general transcriptional machinery on target promoters (Heitzer and DeFranco, 2006; Perissi *et al.*, 2004). Histones can be modified by acetylation, phosphorylation, methylation, ubiquitination, and Sumoylation (Glass *et al.*, 1997; Roelfsema *et al.*, 2005), and the functions of acetylation and phosphorylation have been well characterized. For example, some co-activators in the 160 family have histone acetyltransferase (HAT) activity and the site-specific acetylation of histone leads to decompacting repressive chromatin, which makes chromatin more accessible to other transcriptional regulators and transcriptional machinery (Jenuwein and Allis, 2001). SWI/SNF, components of a co-activator complex, can also serve as an ATP-dependent chromatin modification complex and involved in further chromatin modification, which is responsible for general transcriptional factor recruitment (Belandia *et al.*, 2002). In addition to involvement in the chromatin remodeling events during the transcriptional activation process, co-activators also play important roles during transcription, including participating in the pre-initiation complex assembly, transcriptional elongation and even

mRNA processing (Wolf *et al.*, 2008). The transcription at the promoters of nuclear receptor target genes is cyclic due to the dynamic assembly/disassembly/reassembly of the co-activators complex (Shang *et al.*, 2000). In addition to modifying histones, co-activators themselves can also undergo posttranslational modifications, resulting in the changes of either activities or stabilities of co-activators (Wu *et al.*, 2007). Phosphorylation is one of the well-characterized posttranslational modifications of co-activators, which not only determines their intracellular activity, but also is important for their proper functions (Wu *et al.*, 2004). Furthermore, phosphorylation can also enhance the subsequent ubiquitination of co-activators, which usually lead to more transcriptionally active co-activators. Monoubiquitination of co-activators can regulate their transcriptional regulatory activity, while progressive polyubiquitination eventually results in proteasome degradation (Lonard and O'Malley B, 2007). Consistence with above observations, it has been found that components of the ubiquitin-proteasome system (UPS) are present in the co-activator complex and involved in regulating the co-activator activities and maintaining the dynamic association and dissociation of the co-activator complex with target promoters by actively mediating the degradation of chromatin-bound receptors and/or co-activator complex (Rosenfeld *et al.*, 2006).

The nuclear receptor co-repressor complex, which is necessary for the nuclear receptor-mediated repression, consists of nuclear receptor co-repressor (N-CoR), the silencing mediator of RAR and TR (SMRT), histone deacetylase (HDAC), transducin β-like 1 (TBL1), transducin β-like 1-related protein (TBLR1) and GPS2. N-CoR is a 270kDa protein, which is associated with TR-RXR heterodimers and can mediate ligand-independent inhibition of target gene transcription by these receptors (Horlein *et al.*,

1995). SMRT, a homologous protein of N-CoR, is a receptor-interacting factor and required for retinoid and thyroid hormone receptor-mediated repression (Chen and Evans, 1995). Even though N-CoR and SMRT are responsible for some nuclear receptor-mediated transcriptional repression, HDAC activity is important and involved in this repression. The classical HDAC falls into three classes in mammals, namely class I, class II and class IV. The class I HDACs (HDAC1, 2, 3, and 8) are most closely related to the yeast transcriptional regulator RPD3. Class II HDACs (HDAC4, 5, 6, 7, 9, and 10) shares domains with similarity to HDA1, another deacetylase found in yeast (Bjerling *et al.*, 2002; Fischle *et al.*, 2002). HDAC11 is the unique member of class IV HDACs (Gao *et al.*, 2002). Although there are many HDACs, HDAC3 is of particular interest in the nuclear receptor co-repressor complex based on reported data. (1) HDAC3 is found in a tight complex with SMRT and/or N-CoR (Guenther *et al.*, 2000; Li *et al.*, 2000; Wen *et al.*, 2000; Zhang *et al.*, 2002), and its enzyme activity is completely dependent on association with the deacetylase activating domain (DAD) of N-CoR or SMRT (Guenther *et al.*, 2001; Zhang *et al.*, 2002). (2) The N-CoR/HDAC3 complex is required for repression by thyroid hormone receptor. The endogenous N-CoR, TBL1, and HDAC3, but not other HDACs, are recruited to a stably integrated reporter gene repressed by unliganded TR, which indicates HDAC3 is critical for repression by multiple nuclear receptors and the N-CoR/HDAC3 complex plays a unique and necessary role in TR-mediated gene repression (Ishizuka and Lazar, 2003; Yoon *et al.*, 2003). TBL1, TBLR1, and GPS2 are additional components of the N-CoR/SMRT complex. TBL1 and TBLR1 are F box/WD40 proteins, which are usually important components in RING finger domain E3 ubiquitin ligases and responsible for specific substrate recognition. Even

though they are in the co-repressor complex, TBL1 and TBLR1 are surprisingly required for gene activation by liganded nuclear receptors since they are capable of mediating the co-repressor/co-activator exchange through the ligand-dependent recruitment of the ubiquitin/19S proteasome complex (Perissi *et al.*, 2004).

The co-repressor/co-activator exchange model

Here, we use RARα-mediated retinoic acid signaling as an example to illustrate the co-repressor/co-activator exchange model (Figure 1.4 RA signaling pathway and co-repressor/co-activator exchange model). Usually, RARα heterodimerizes with the retinoid X receptor, RXR, and induces repression or activation of target genes in response to ligands. In the absence of ligand, RARα/RXR are found primarily in the nucleus and bind to specific DNA sequences or RA response elements (RARE), they associate with the nuclear receptor co-repressor N-CoR, or SMRT, which repress the transcription of target genes by forming repression complexes with enzymatic activities such as histone deacetylation. Upon ligand RA binds to RARα, the receptors will undergo conformational changes, which cause the dissociation of the co-repressors and the recruitment of the co-activators. These co-activators complexes have histone acetyltransferase (HAT) activities, which lead to de-compacting repressive chromatin. Once repressive chromatin has been de-condensed, the receptors become able to recruit the transcription machinery by their association with the mediator complex, which facilitate the entry of transcriptional machinery to the promoter and initiation transcription. TBLR1 specifically serves as a co-repressor/co-activator exchange factor by recruiting the 19S proteasome, and this function is required for the dismissal and subsequent degradation of co-repressor N-CoR, SMRT, HDAC3 and required for the

subsequent recruitment of the co-activator complex. In the presence of ligand, most nuclear receptors also undergo proteasome degradation, which might also provide a mechanism to control the magnitude and the duration of ligand-mediated transcription (Dennis *et al.*, 2001; Perissi *et al.*, 2004).

Although gene transcription and ubiquitination-mediated protein degradation seem two totally unrelated processes, an increasing amount of evidence shows that the ubiquitin proteasome system links to gene transcription closely. This link is most significant in the nuclear receptor (NR)-dependent transcription and degradation. MG132, a proteasome inhibitor, can block RA-induced RAR degradation and cause RAR accumulation in a murine lung alveolar carcinoma cell line. But at the same time, MG132 can attenuate the RAR trans-activation even in the presence of increasing amount of RAR (Andela and Rosier, 2004). Similar observations have been reported for other NRs, including estrogen receptor α (ERα) (Lonard *et al.*, 2000), progesterone receptor (PR) (Lange *et al.*, 2000), androgen receptor (AR) (Lin *et al.*, 2002), thyroid hormone receptor (TR) (Dace *et al.*, 2000), and retinoic acid receptor α (RARα) (Zhu *et al.*, 1999). However, not all NR-regulated genes require this degradation function, since the proteasome inhibition enhances transcriptional activity of glucocorticoid receptor (GR) (Deroo *et al.*, 2002; Wallace and Cidlowski, 2001). Another group found that SK-N-DZ cells (a neuroblastoma cell line), which have a defect in RARα down regulation, expressed relatively high levels of retinoic acid receptor α (RARα) and underwent ATRA-induced cell death. While NH12 and SK-N-SH cells (a neuroblastoma cell line), which have relative normal RARα down-regulation, are refractory to ATRA treatment, and treatment with a proteasome inhibitor caused RARα accumulation and dramatically

increased ATRA-induced cell death (Nagai *et al.*, 2004). The reasons for the different behaviors of the nuclear receptors in different cell types in response to ligands and proteasome inhibitors are not fully elucidated. Some additional factors might be involved in these processes.

Ski is a part of co-repressor complex for nuclear receptor signaling pathway

Ski and SnoN form complexes with N-CoR, SMRT, HDAC3 and mSin3, and are involved in transcription regulation of several nuclear receptors. Ski can interact with RARα and block its transactivation activity (Dahl *et al.*, 1998a; Ritter *et al.*, 2006b). Furthermore, Ski can also interact with vitamin D receptor and negatively regulated vitamin D-induced transcription, which is N-CoR dependent (Ueki and Hayman, 2003b).

The interaction of Ski with other transcription factors

The members of Ski/SnoN family are involved in many events and their primary function is to regulate transcription. They have to function through interaction with other cellular partners since they do not have catalytic activities and they are unable to bind DNA directly. Besides TGF-β and nuclear receptor signaling pathways, Ski/SnoN can negatively regulate many other signaling pathways by interacting with many proteins, including transcription factors, such as Gata1(Ueki *et al.*, 2004), PU.1 (Ueki *et al.*, 2008), pRb (Tokitou *et al.*, 1999), and others.

Ski and GATA1 interaction inhibits erythroid differentiation

GATA-1, a zinc finger transcription factor, was first identified by its ability to interact with a consensus element (T/A)GATA(A/G) in regulatory region of the globin gene and erythroid and megakaryocytic-specific genes (Evans and Felsenfeld, 1989; Orkin, 1992; Tsai *et al.*, 1989). GATA-1 plays a key role in erythroid development and

targeted mutation of GATA-1 can block the erythroid differentiation (Pevny *et al.*, 1991). It has been reported that Ski-GATA-1 interaction can displace GATA-1 occupancy from the GATA-binding motif on the promoter and enhancer regions of erythroid specific genes, which leads to the repression of GATA-1 mediated transcriptional activation (Ueki *et al.*, 2004). Since Ski has been shown to be involved in the development of highly malignant erythroleukemia (Larsen *et al.*, 1992), the study above provided a possible mechanism by which Ski can block normal erythroid differentiation and contribute to the development of erythroleukemia.

Ski and PU.1 interaction can negatively regulate macrophage differentiation

PU.1, another lineage-specific transcription factor, is important for the development of multiple lineages of hematopoietic cells and the levels of PU.1 are critical determinants for the specification of multiple cell fates (Dahl and Simon, 2003). For example, in a granulocyte-macrophage progenitor, high levels of PU.1 can direct macrophage differentiation, while low levels direct granulocyte differentiation (Dahl and Simon, 2003). Ski is one of PU.1 antagonists. Ski can interact with PU.1 and inhibit PU.1-dependent transcriptional activation. Furthermore, by being in the same complex with PU.1, Ski negatively regulates PU.1-mediated macrophage differentiation. The mechanism underline this phenomena is that Ski can recruit histone deacetylase 3 (HDAC3) to PU.1 bound DNA, which is different mechanistically from the inhibitory effects of Ski on GATA-1 (Ueki *et al.*, 2008).

Posttranslational modifications of the Ski and SnoN proteins

There are several levels of regulation responsible for maintaining the proper functions of proteins, one of which is posttranslational modification. Ski and SnoN can

regulate many signaling pathways and are involved in various events, which relate to both vertebrate development and cancer progression. The proteins themselves are also tightly controlled at different levels, including the expression levels and a variety of modifications responsible for their proper functions.

Protein ubiquitination and degradation

Both Ski and SnoN undergo degradation in response to TGF-β signaling (Stroschein *et al.*, 1999; Sun *et al.*, 1999). TGF-β induced Ski degradation might be cell type specific. In mink lung epithelial cells with stably expressed Ski, TGF-β treatment leads to decreased levels of Ski, and pretreating cells with the proteasome inhibitor, MG132, can completely block this degradation. This observation suggests that TGF-β might mediate Ski proteasome degradation (Sun *et al.*, 1999). However, this is not the case in some cell lines. For example, two- hour TGF-β treatment could not induce significant degradation of the Ski protein in primary culture cells from human ovarian surface epithelium (HOSE) and ovarian carcinomas (CSOC), suggesting Ski might function differently from that in mink lung epithelial cells or it is not a co-repressor for TGF-β pathway in these two cell lines (Baldwin *et al.*, 2003). TGF-β-induced Ski degradation is cell type dependent and this degradation occurs in metastatic breast cancer and melanoma cell lines (Le Scolan *et al.*, 2008; Nagano *et al.*, 2007). Furthermore, Arkadia, a nuclear protein with a characteristic RING domain in its C terminus, could act as the E3 ubiquitin ligase responsible for TGF-β-mediated Ski degradation (Nagano *et al.*, 2007).

In addition to TGF-β-dependent Ski degradation, cell cycle-dependent degradation of Ski has also been observed both in vitro and in vivo. The Ski protein level

varies throughout the cell cycle with lowest at G0/G1, and highest at G2/M in MG63 cells stably expressing Ski (Marcelain and Hayman, 2005). Similarly, researchers used xenopus egg extracts prepared from the interphase or mitotic phase of the cell cycle to study the Ski degradation throughout the cell cycle and found Ski degraded in interphase extracts and is relatively stable in mitotic extracts (Macdonald *et al.*, 2004). Cdc34, a ubiquitin-conjugating enzyme, is responsible for the cell cycle-dependent Ski degradation, which is further confirmed by the fact that Ski can be stabilized and has stronger inhibitory effect on TGF-β signaling in the presence of dominant-negative Cdc34 (Macdonald *et al.*, 2004).

The study on TGF-β-mediated SnoN proteasome degradation is more complete and several E3 ubiquitin ligases are responsible for its ubiquitination. Smurf2 (Smad ubiquitin regulatory factor) is a HECT domain-containing E3 ubiquitin ligase and can associate with SnoN via Smad2 in the presence of TGF-β signaling, which allows the HECT domain of Smurf2 to target SnoN for ubiquitin-proteasome degradation (Bonni *et al.*, 2001). Another E3 ubiquitin ligase responsible for SnoN degradation is anaphase-promoting complex (APC) (Stroschein *et al.*, 2001; Wan *et al.*, 2001). Unlike Smurf2, which functions through Smad2, APC mediates SnoN degradation via Smad3. CDH1 in the APC complex, serving as a substrate-specific activator, can recognize and bind to SnoN in the presence of Smad3, resulting in SnoN proteasome degradation (Wan *et al.*, 2001). Like the effect of Arkadia on Ski degradation, Arkadia can also mediate SnoN degradation and enhance TGF-β signaling (Nagano *et al.*, 2007).

The SnoN protein can also be regulated throughout the cell cycle, but there is a disagreement about the expression pattern at different phases. Some data suggest that

SnoN expression is higher at G1 (Mimura *et al.*, 1996; Pearson-White and Crittenden, 1997), while others indicate it should be higher in G2/M and the APC complex might be responsible for the cell cycle-dependent SnoN degradation (Wan *et al.*, 2001).

More importantly, three critical lysine residues in the C-terminal domain of SnoN have been identified, K440, K446, K449. The polyubiquitin chains are attached on these sites, which leads to SnoN being recognized and degraded by the proteasome system (Stroschein *et al.*, 2001). Even though Ski is ubiquitinated and degraded via ubiquitin-proteasome system, the lysine residues responsible for the polyubiquitin chains formation on Ski have not been characterized.

Ubiquitin-dependent degradation of Ski and SnoN may play important roles in both physiological and pathological processes since they are negative regulators for many signaling pathways, which are involved in cell proliferation, differentiation, survival and death. The tight regulation of Ski and SnoN protein levels can help to maintain the balance of these events, and breaking this balance is often associated with tumor formation. Growth inhibitory response to TGF-β signaling is important in rapidly renewing epithelial cells and defective TGF-β signaling is a common feature of epithelial cancer, such as esophageal cancer. Both Ski and SnoN have been found to be up-regulated in esophageal cancer (Akagi *et al.*, 2008; Fukuchi *et al.*, 2004; Villanacci *et al.*, 2008), which implicates impaired TGF-β mediated Ski and SnoN degradation may contribute to the tumor development. TGF-β can no longer mediate SnoN degradation, which also happens in growth inhibition-resistant esophageal cancer cells. Furthermore, growth inhibition sensitive OE33, an adenocarcinoma cell line, became resistant to TGF-β-induced growth arrest when transfected with SnoN mutants that lacking the three

critical lysine residues for polyubiquitin chains formation (Edmiston *et al.*, 2005). All these data suggest that the deregulation of Ski and SnoN levels might be associated with cell transformation and tumor formation.

Other modifications of Ski and SnoN

Besides ubiquitination and subsequent proteasome degradation, both Ski and SnoN are phosphorylated. SnoN can be phosphorylated on S115, S117 and/or T119 by TGF-β activated kinase (TAK1), which is a member of mitogen activated kinase kinase kinase (MAPKKK) family. TAK1 interacts with and phosphorylates SnoN in the nucleus, which is essential for TGF-β-dependent SnoN ubiquitination and proteasome degradation (Kajino *et al.*, 2007). Even though Ski has also been shown to be phosphorylated exclusively on serine residues, the exact phosphorylation sites on Ski have not been identified (Sutrave *et al.*, 1990a). Furthermore, Ski phosphorylation is regulated throughout the cell cycle, which is up-regulated in mitosis, and the Cdc2-Cyclin B kinase complex may be responsible for Ski phosphorylation during mitosis (Marcelain and Hayman, 2005).

Small ubiquitin-like modifier (SUMO) can also modulate the function of SnoN. Sumoylation of SnoN occurs on lysines 50 and 383 by SUMO E3 ligase PIASs (Hsu *et al.*, 2006; Wrighton *et al.*, 2007), which is independent of SnoN ubiquitination and has no effects on protein stability. However, sumoylation results in enhanced repressive effects of SnoN on myogenesis (Hsu *et al.*, 2006; Wrighton *et al.*, 2007).

The ubiquitin-proteasome system

Ubiquitin-proteasome system (UPS) is responsible for a variety of normal and abnormal intracellular protein degradation, including damaged, misfolded or improperly

translated proteins and regulatory proteins whose degradation happens only under certain conditions and are required for proper response and function of the cells (Bogyo *et al.*, 1998). UPS-mediated proteolysis is involved in many processes, such as proper cell cycle progression (Koepp *et al.*, 1999), antigen presentation (Rock and Goldberg, 1999), transcriptional regulation (Leung *et al.*, 2008), and immune and inflammatory responses (Wang and Maldonado, 2006). The deregulation of the UPS-mediated proteolysis is associated with many disorders, including neurodegenerative diseases (Ciechanover and Brundin, 2003; Ding and Shen, 2008; Paul, 2008) and various kinds of cancer development.

There are two major steps involved in protein degradation via UPS, including the covalent attachment of multiple ubiquitin molecules to the proteins substrates and the formation of polyubiquitin chains, and the recognition and degradation of the polyubiquitinated protein by the 26S proteasome complex (Hershko, 1983) (Figure 1.5 Ubiquitin-Proteasome System). There are at least three enzymes involved in the first step: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). Ubiquitin (Ub), a polypeptide with 76 amino acids, is activated by an E1 in an ATP-dependent manner and covalently linked through a thioester bond to an active-site cysteine in the E1. Then, the activated ubiquitin is transferred from the E1 to E2. Finally, ubiquitin is covalently linked by an isopeptide bond to a lysine in the target proteins or to another ubiquitin molecule that has already been linked to the target protein, which is accomplished by an E3. In the second step, the polyubiquitinated protein is recognized and unfolded by the 26S proteasome and destroyed in an ATP-dependent manner (Pickart, 2001).

The E3 appears to play the critical role in recognizing, binding specifically to, and recruiting target proteins for ubiquitination. There are many E3 ubiquitin ligases, which can be divided into four classes: HECT domain E3; U box and PHD finger E3, monomeric RING finger domain E3 and multisubunit RING finger domain E3 (Passmore and Barford, 2004) (Figure 1.6 Classification and structural model of E3 ubiquitin ligase). HECT E3 is characterized by its unique HECT domain, which is a large C-terminal module of about 350 residues and was originally identified in the E6-associated protein (E6-AP) (Huibregtse *et al.*, 1995). The HECT domain consists of a larger N-terminal lobe, which is responsible for E2 binding, and a smaller C-terminal lobe, which has an active-site cysteine residue and is responsible for loading ubiquitin on themselves and then transferring the ubiquitin to substrates (Huang *et al.*, 1999; Verdecia *et al.*, 2003). Monomeric RING finger domain E3 and multisubunit RING finger domain E3 are the largest family of E3 and characterized by the RING finger consensus sequence (Really Interesting New Gene), which has eight conserved cysteines and histidines with two coordinating Zn ions in a unique "cross-brace" arrangement (Fang *et al.*, 2003; Freemont, 2000; Passmore and Barford, 2004; Saurin *et al.*, 1996). Single-subunit RING E3s are the proteins that are responsible for both E2 interaction and substrates recognition. While the multisubunit E3 is a complex, in which the RING finger protein is responsible for E2 binding and other components have the ability to recognize substrates. These multimeric complexes can be further divided into two types: cullin-based complexes, and the APC/C (anaphase promoting complex/cyclosome), which contains the cullin-like protein APC2 (Pickart, 2001; Vodermaier, 2004). Two RING finger-related domains, U box and PHD finger, also have the potential to confer E3 ubiquitin ligase activity. U box consensus

sequence can assume RING finger-like structure by salt bridges and hydrogen bonds instead of by conserved Zn-coordinating residues for RING finger (Aravind and Koonin, 2000). Some U box proteins don not have independent E3 activity, but can facilitate HECT domain E3 during target protein ubiquitination (Koegl *et al.*, 1999). PHD finger domain is a small motif of approximate 60 amino acids, which is characterized by seven cysteines and a histidine and arranged spatially in a C4HC3 consensus (Aasland *et al.*, 1995). Even though not all the PHD finger domain proteins have E3 activity, PHD finger domain rather than the RING finger domain is required for some proteins' ability to mediate ubiquitination (Coscoy *et al.*, 2001; Song *et al.*, 1994).

Once substrates are recognized by E3 and polyubiquitinated, they can be further recognized by the 26S proteasome and undergo ATP-dependent unfolding and degradation. The 26S proteasome consists of three subcomplexes: one 20S proteasome and two 19S regulatory particles. The 19S regulatory particles are responsible for recognizing polyubiquitinated proteins, unfolding substrates to enter to 20S proteasome, which has the catalytic sites and control the substrate proteolysis (Wang and Maldonado, 2006).

In addition to mediate protein degradation, ubiquitination can influence target proteins functions. For example, monoubiquitination can be a signal for intranuclear trafficking (Garcia-Higuera *et al.*, 2001). It is well known that polyubiquitin chains is a signal for substrate destruction by 26S proteasome, but depending on the lysines that polyubiquitin chain is attached, target proteins may have different fates. For example, polyubiquitin chain linked to Lys63 on the substrate can activate kinase property rather

than proteasomal proteolysis, which is usually triggered by polyubiquitin chain attached on Lys48 (Deng *et al.*, 2000).

Since the UPS can affect lots of cellular processes, from gene transcription and DNA repair to cell cycle and apoptosis by controlling the levels, activities, and locations of various cellular proteins, the deregulation of UPS has been implicated in the development of various diseases, including cancers (Paul, 2008; Yang *et al.*, 2009). Fully understanding the UPS could provide more information about the nature of diseases and help to develop approaches that are more effectively therapeutic.

Summary and dissertation aims

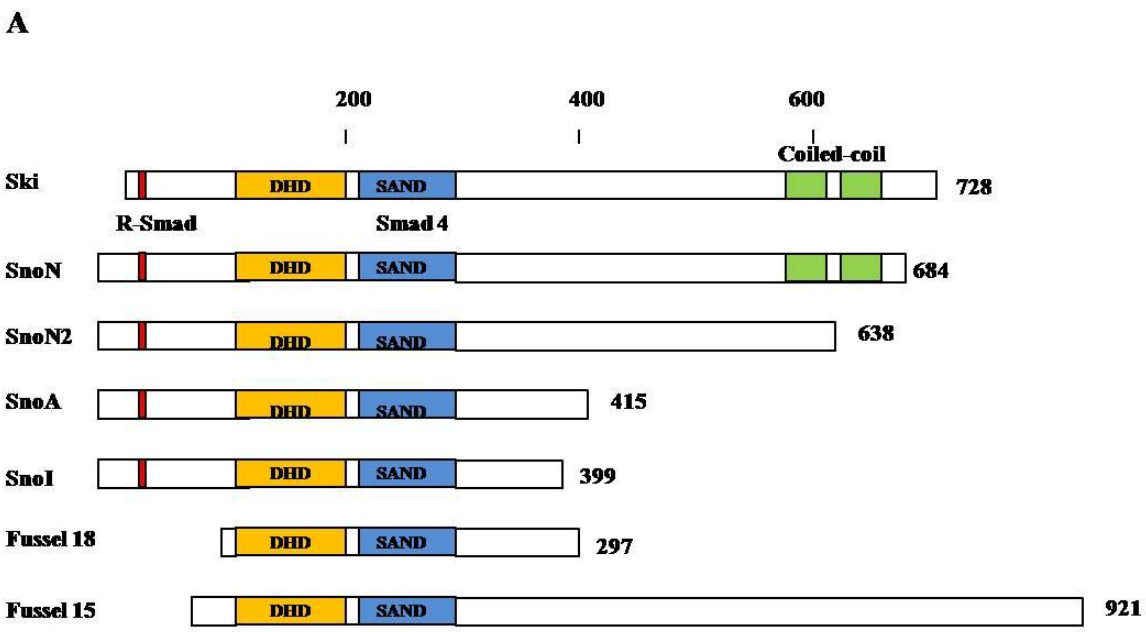
Our lab has been working on the Ski protein over the past years and trying to understand how the Ski protein can be involved in so many signaling pathways and what mechanisms are involved in the various functions of Ski. We have made significant progresses regarding to the role of Ski in transcriptional regulation and the regulation of Ski in cell cycle. I am particularly interested in the role of Ski in nuclear receptor signaling. We found that Ski can interact with RARα and inhibit RA induced transcriptional activation, and this interaction seems to play a role in leukemia development (Dahl *et al.*, 1998a). This observation is further supported by the fact that two-three fold up-regulation of Ski protein in a subset of human acute myeloid leukemia, AML, and this increased expression correlates with bad prognosis and resistance to ATRA-induced differentiation therapy (Ritter *et al.*, 2006b). However, the detailed mechanism of Ski's repressive effects on RARα-mediated transactivation is still unclear. This dissertation aimed to address the mechanism of Ski's repressive effects on RA signaling pathway and its role in the co-repressor/co-activator exchange process. Unlike

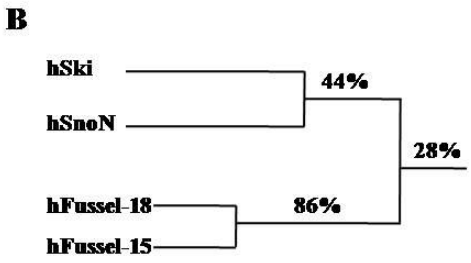
other components in the co-repressors complex, Ski associated with RARα even in the presence of ligand RA, which makes Ski more interesting in RA signaling pathway. We will describe the role of Ski in the degradation of proteins involved in the co-repressor complex. In addition, we will further identify the possible E3 ligase whose activity is required for the degradation of these proteins degradation and look at the effects of Ski on the E3 ligase activity.

Figure 1.1 Schematic diagram of the Ski/Sno family

This diagram represents the members of the Ski/Sno family.

A. Domain structures of Ski/Sno family The number of amino acids for each protein is indicated on the right. R-Smads in the N-terminal regions of Ski, SnoN, SnoN2, SnoA and SnoI are responsible for phosphorylated, trimeric form of Smad 2/3 interaction. The DHD (Dachshund homology domain) is a compact, globular domain and can mediate the interaction between the Ski/SnoN family and other proteins, such as N-CoR, SKIP. SAND (named after Sp100, AIRE1, NucP41/75, and DEAF1) is a Smad 4 binding domain. The C-terminal regions of Ski and SnoN are required for Ski homodimer formation and Ski-SnoN heterodimerization.
B. Homology tree of entire protein sequences The Ski and SnoN proteins share an about 44% amino acid homology. Fussel-15 and Fussel-18 proteins show 86% homology to each other. Fussel-15 and Fussel-18 proteins share an overall 28% amino acid homology with Ski and SnoN (Modified from Arndt *et al.,* 2007).
C. Homology tree of homology domains The Ski/Sno family shares similar domain structures in their N-terminal regions (Adopted from Arndt *et al.,* 2007).


A
200
400
600
Coiled-coil
Ski
DHD
SAND
728
R-Smad
Smad 4
SnoN
684
SnoN2
638
SnoA
415
SnoI
399
Fussel 18
297
Fussel 15
921


B
hSki
hSnoN
hFussel-18
hFussel-15
44%
28%
86%

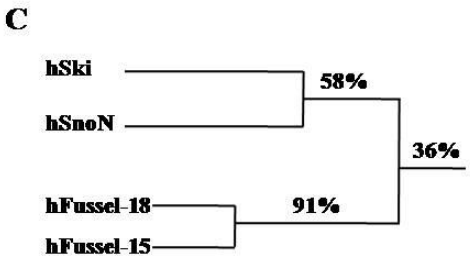
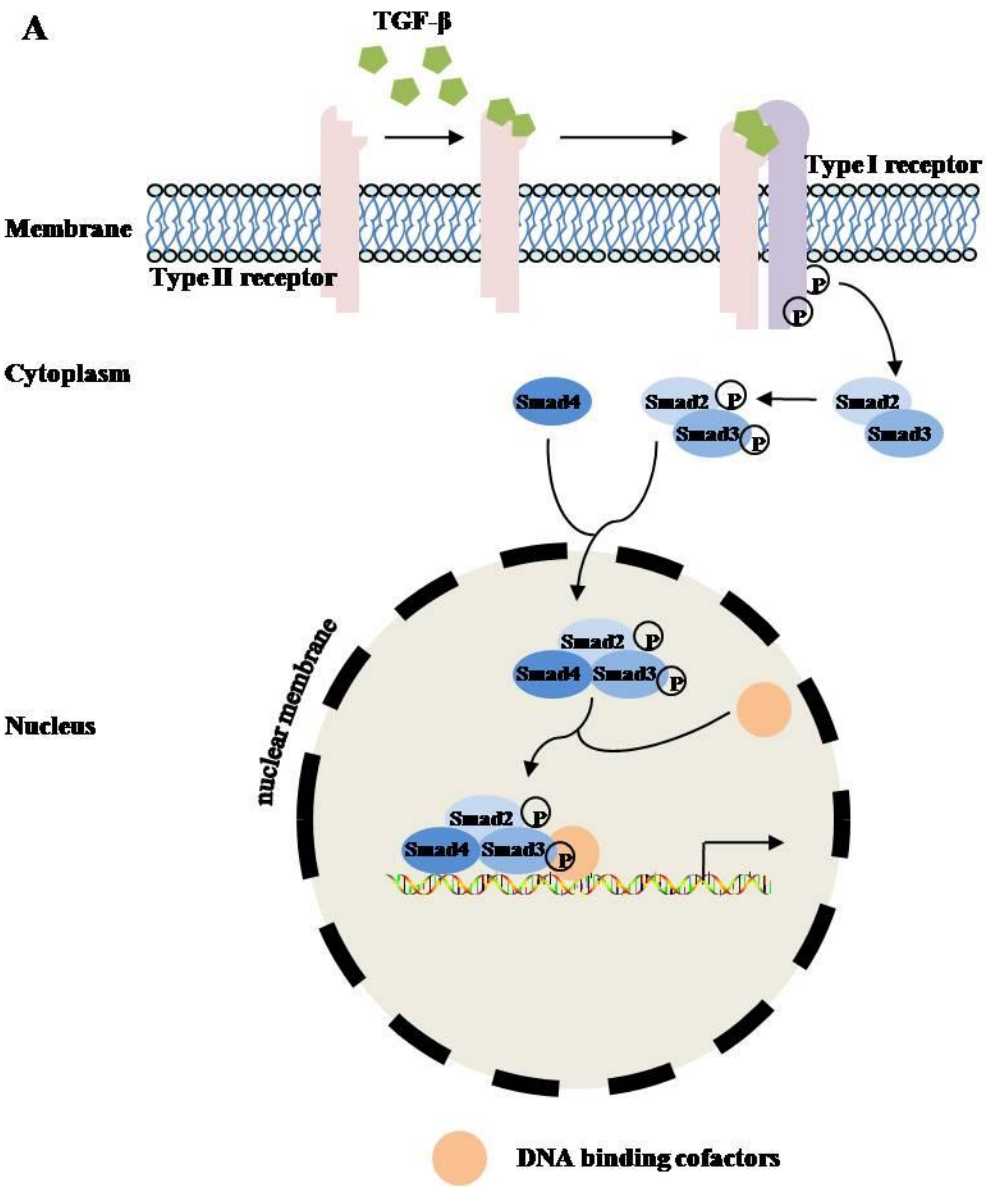

C
hSki
hSnoN
hFussel-18
hFussel-15
58%
36%
91%

Figure 1.2 Ski/SnoN and TGF-β pathway

A. TGF-β signaling pathway Active TGF-β can bind to type II and type I serine/threonine kinase receptors on the cell membrane and activate type II receptor, then the activated type II receptor can transphosphorylate and activate type I receptor kinase, which can further phosphorylate receptor-regulated Smads (R-Smads), Smad2/3. The phosphorylated R-Smads, Smad2 and Smad3, form a heteromeric complex with Co-Smad, Smad4 and translocate into the nucleus. Here the Smad2/3-Smad4 heteromers interact with various transcription factors, transcriptional co-activators and lead to transcriptional activation of target genes.
B. Possible mechanisms involved in the inhibitory effects of Ski/SnoN on TGF-β pathway. There are several possible mechanisms involved in this repression as showed in the figure. (1) High level of Ski might interfere with the phosphorylation of Smad2 by activated type I receptor kinase and prevent subsequent signaling transduction. (2) Ski might interrupt the Smad2/3-Smad4 heteromer formation by competing with phosphorylated Smad2 for binding to Smad4 and prevent R-Smad from translocation into the nucleus. (3) Smad2/3-Smad4 heteromeric complexes still form, but they are inactive and stabilized on the promoter region of the TGF-β target genes to prevent further binding of newly synthesized and active Smads complex or high expression of Ski and SnoN may inhibit the binding of Smads complex to transcriptional co-activator complex and recruit transcriptional repressor complex.


A
TGF-β
Type I receptor
Membrane
Type II receptor
P
P
Cytoplasm
Smad4
Smad2
P
Smad3
P
Smad2
Smad3
nuclear membrane
Smad2
P
Smad4
Smad3
P
Nucleus
Smad2
P
Smad4
Smad3
P
DNA binding cofactors

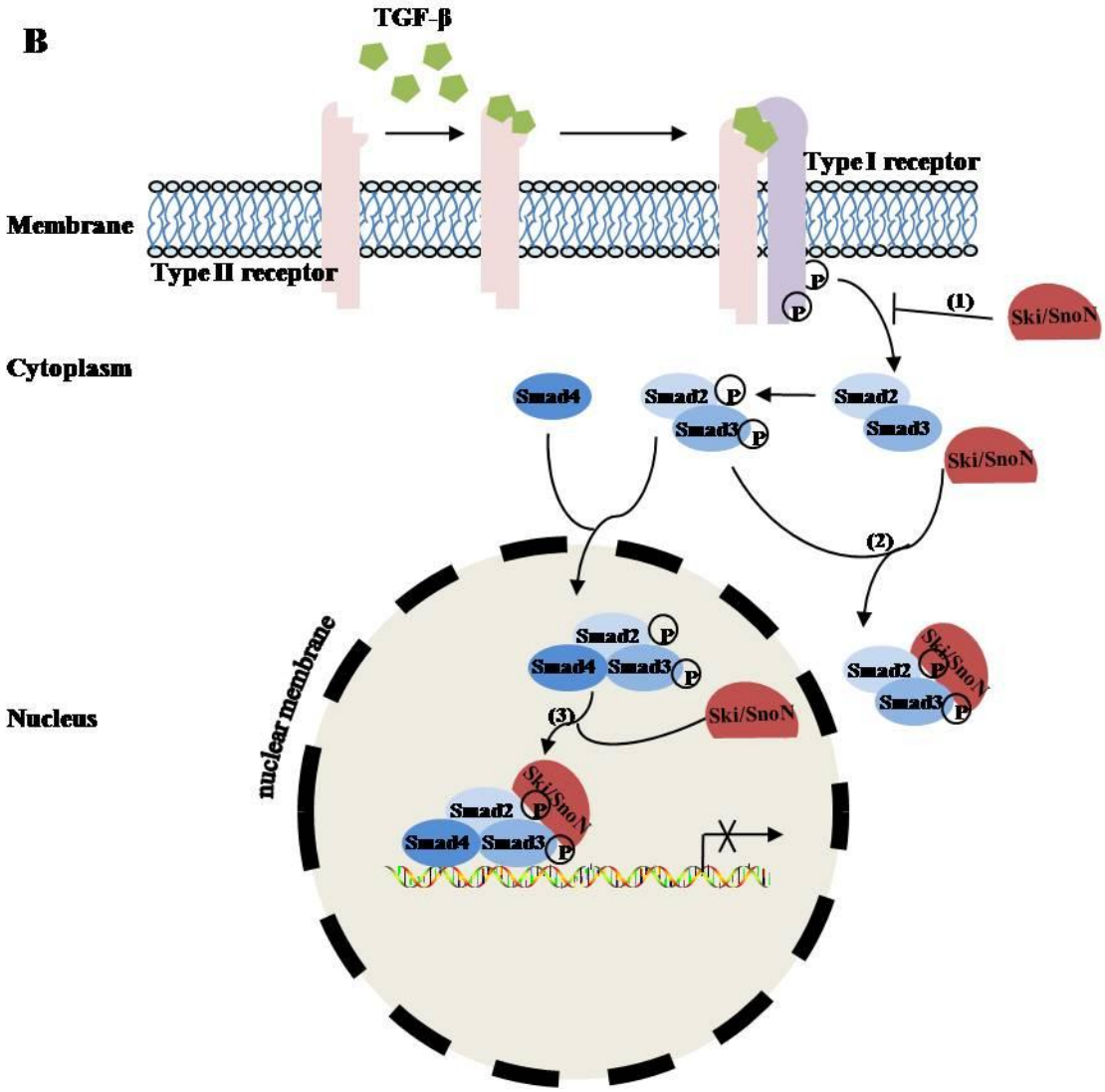

B
TGF-β
Type I receptor
Membrane
Type II receptor
(1)
Ski/SnoN
Cytoplasm
Smad4
Smad2
Smad3
P
Ski/SnoN
(2)
nuclear membrane
Smad2
Smad4
Smad3
P
(3)
Ski/SnoN
Nucleus

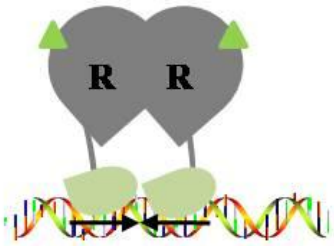
Figure 1.3 Classification and structure domains of nuclear receptors

A. Nuclear receptors can be divided into four classes according to the mode that they bind to their DNA elements. Class 1: The receptors are cytoplasmic and can be activated by ligand in the cytoplasm. The activated receptors translocate into the nucleus and bind to specific palidromic response elements in the promoter regions of target genes as homodimers. Class 2: These receptors are nuclear and recognize a direct repeat response element in the absence of ligand as heterodimers with retinoic X receptor (RXR). The ligands activate them in the nucleus. Class 3: Orphan receptors bind to DNA as monomers. Class 4: Orphan receptors bind to DNA as dimers with RXR. Even though class 3 and class 4 are orphan receptors, and ligands for some of orphan receptors have been identified.

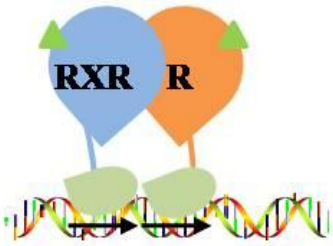
B. The full-length nuclear receptor proteins can be divided into six regions (A-F). There is a ligand-independent transcriptional activation function domain (AF-1) in the variable N-terminal region (A/B region). All of the nuclear receptors contain a central DNA binding domain (DBD) (region C), which is the most conserved domain and responsible for docking receptors to the DNA response element upstream of target genes. Ligand binding domain (LBD) (region E) is in the C-terminal half of the receptor and performs a variety of important functions, such as ligand-regulated transcriptional activation function (AF-2), providing an interior binding pocket required for the ligand binding, and containing dimerization motifs for receptor dimerization. There is a hinge (region D) between the DBD and LBD and the complete functions of this part are still elusive. Most receptors also have a variable C-terminal region F.

A

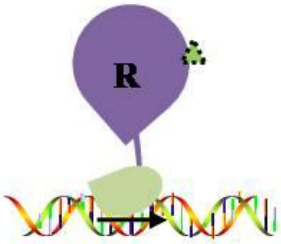
Class 1: Steroid receptors
GR, PR, AR, ER, etc.



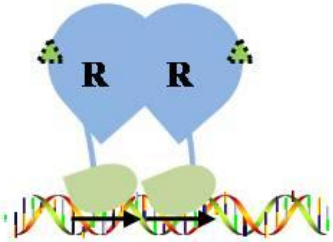
Class 2: RXR heterodimers:
TR, RAR, PPAR, VDR, etc.



Class 3: monomeric orphan receptors



Class 4: Dimeric orphan receptors



B

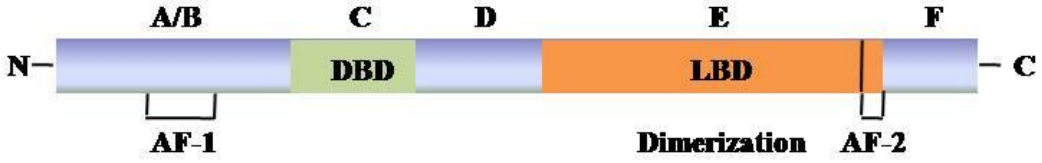


Figure 1.4 RA signaling pathway and co-repressor/co-activator exchange model Based on the model in (Perissi *et al.*, 2004)

In the absence of ligand, RARα and RXR form heterodimer and bind to specific DNA sequences or RA response elements (RARE), they associate with the nuclear receptor co-repressor complex including N-CoR, or SMRT, HDAC3 and other factors (X), which mediate target gene repression. Upon ligand RA binds to RARα, the receptors will undergo conformational changes, which cause the dissociation of co-repressors and the recruitment of co-activator complex. The co-activator complex has histone acetyltransferase (HAT) activities and can recruit and facilitate the transcription machinery to the promoter and initiation transcription. The components (for example TBLR1) in the other factors may mediate subsequent co-repressor complex degradation, which is also required for the recruitment of the co-activator complex. In the presence of ligand, RARα also undergoes proteasome degradation.

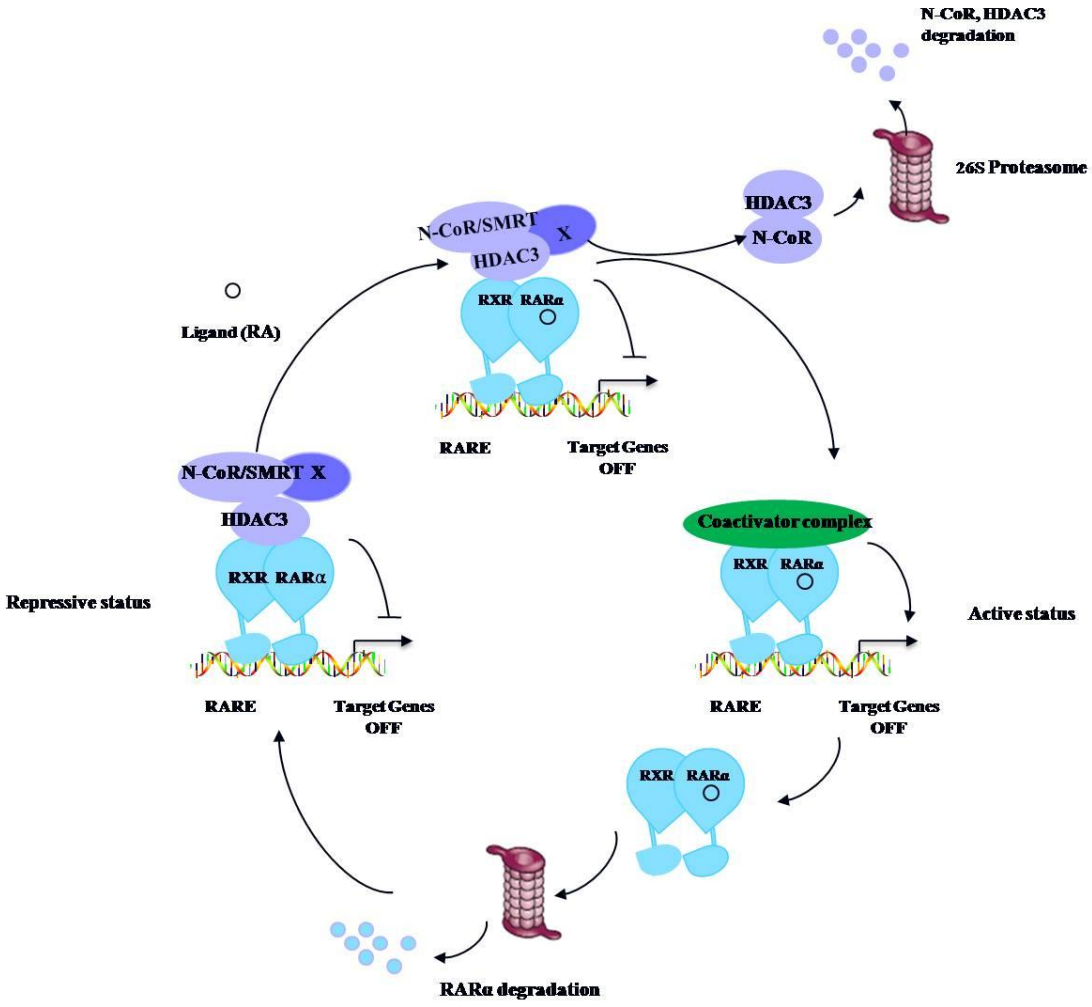

N-CoR, HDAC3
degradation
26S Proteasome
HDAC3
N-CoR
N-CoR/SMRT
X
HDAC3
RXR RARα
Ligand (RA)
RARE
Target Genes
OFF
N-CoR/SMRT X
HDAC3
RXR RARα
Repressive status
RARE
Target Genes
OFF
Coactivator complex
RXR RARα
Active status
RARE
Target Genes
OFF
RXR RARα
RARα degradation

Figure 1.5 the Ubiquitin-Proteasome System (UPS)

There are two major steps involved in protein degradation via UPS. (1) The covalent attachment of multiple ubiquitin molecules to the proteins substrates and the formation of polyubiquitin chains. (2) The recognition and degradation of the polyubiquitinated protein by the 26S proteasome complex. In the first step, the ubiquitin is activated by an ubiquitin-activating enzyme (E1) in an ATP-dependent step and covalently linked to E1. Then, the activated ubiquitin is transferred from the E1 to ubiquitin –conjugating enzyme (E2). Finally, ubiquitin is covalently linked to the target proteins or to another ubiquitin molecule that has already been linked to the target protein, which is accomplished by an ubiquitin ligase (E3). In the second step, the 26S proteasome recognize and unfold the polyubiquitinated proteins, and destroy them in an ATP-dependent manner.

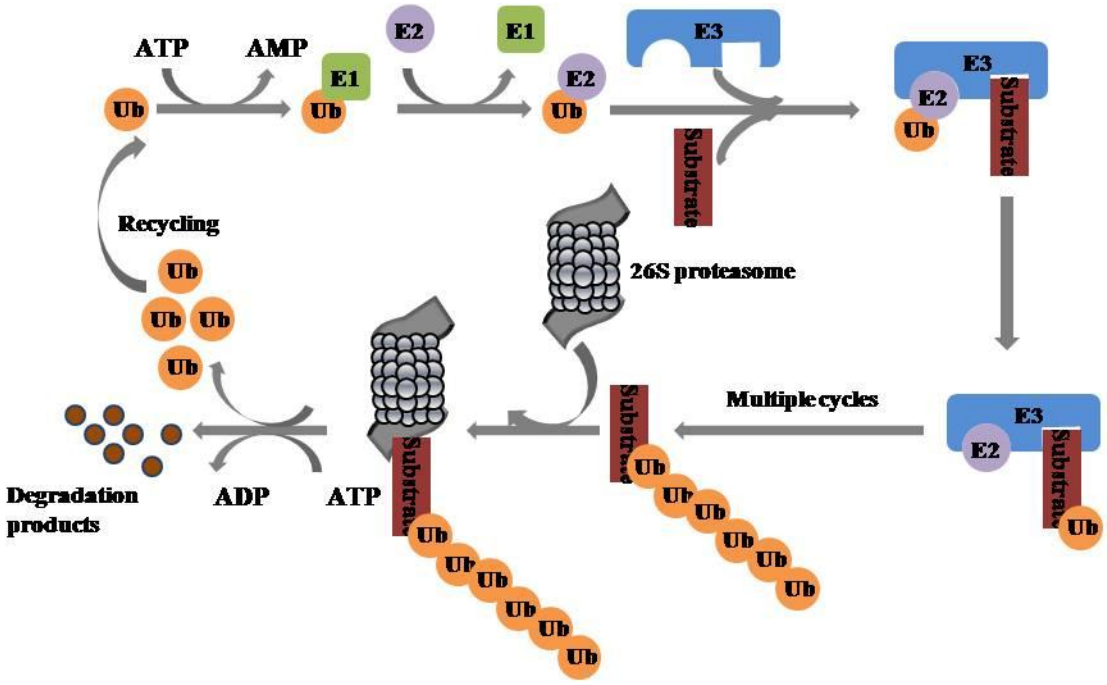

ATP
AMP
E1
E2
E1
E2
E3
E3
E2
Substrate
Ub
Recycling
26S proteasome
Multiple cycles
Degradation products
ADP
ATP

Figure 1.6 Classification and structure models of E3 ubiquitin ligase (modified figure based on (Buschhorn and Peters, 2006; Kipreos, 2005))

Four classes of E3 ubiquitin ligases

A. HECT domain E3 ligase These E3s are unique since they receive Ub from E2 and then transfer Ub to the substrates. The N-terminal lobe of HECT domain is required for E2 binding, and the C-terminal lobe is responsible for receiving Ub and transferring it to the substrates.

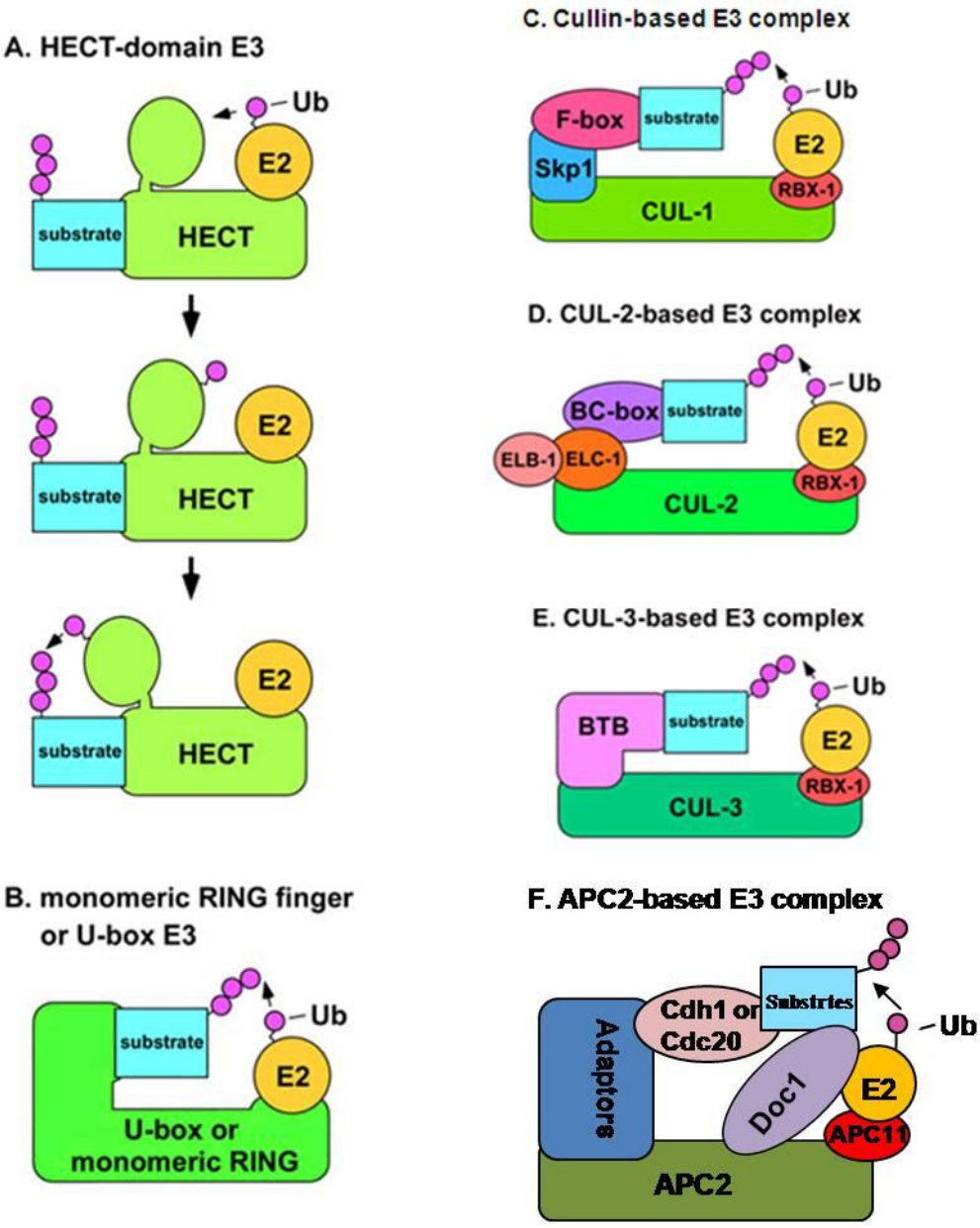
B. U box or monomeric RING finger E3 ligase These E3 ligases are capable of E2 binding and substrates recognition. Then, they can mediate ubiquitination of substrates.

C. Cullin-1 (CUL-1) based multisubunit RING finger domain E3 ligases, also called SCF complex. The N-terminus of CUL-1, acting as a scaffold protein, is responsible for adaptor Skp1 binding, and Skp1 further associates with F-box protein, which can recognize specific substrates. The C-terminus binds the RING finger protein Rbx1, which can interact with E2.

D. Cullin-2 (CUL-2) based multisubunit RING finger domain E3 ligases. Similar to CUL-1 based E3. In this complex, ELC-1 serves as adaptor protein and binds to N-terminus of CUL-2, BC-box protein binds to this adaptor and has substrates recognition function.

E. Cullin-3 (CUL-3) based multisubunit RING finger domain E3 ligases. The N-terminus of CUL-3 can bind directly to BTB, which contains component responsible for substrate recognition, no adaptor protein needed.

F. APC2-based multisubunit RING finger domain E3 ligases, also called APC/C (anaphase promoting complex/cyclosome). APC2 acts as a scaffold protein and binds to Apc11, which is responsible for E2 binding, through a cullin-like domain. The substrates contain a D-box or a KEN-box, which can be recognized by an APC/C co-activator, such as Cdh1 or Cdc20. Doc1 is required for ubiquitination of substrates.


A. HECT-domain E3
Ub
E2
substrate
HECT
B. monomeric RING finger or U-box E3
substrate
Ub
E2
U-box or monomeric RING
C. Cullin-based E3 complex
F-box
substrate
Ub
E2
Skp1
RBX-1
CUL-1
D. CUL-2-based E3 complex
BC-box
substrate
Ub
E2
ELB-1
ELC-1
RBX-1
CUL-2
E. CUL-3-based E3 complex
BTB
substrate
Ub
E2
RBX-1
CUL-3
F. APC2-based E3 complex
Adaptors
Cdh1 or Cdc20
Substrates
Ub
Doc1
E2
APC11
APC2

Chapter II

The Ski protein can inhibit ligand induced RARα and HDAC3 degradation in the retinoic acid signaling pathway

A. Introduction

C-Ski is the founding member of a family of proteins that includes itself and Ski-related protein Sno. v-Ski, a truncated form of the cellular homolog c-Ski, was first identified as the oncoprotein encoded by the avian Sloan-Kettering retroviruses and was responsible for their ability to transform avian fibroblasts in vitro (Stavnezer *et al.*, 1981). c-Ski, v-Ski and Sno can all transform avian embryo fibroblasts *in vitro* when they are overexpressed (Boyer *et al.*, 1993). Ski acts as a transcriptional co-repressor by multiple direct and indirect interactions with several distinct repression complexes. These include complexes contain histone deacetylases, N-CoR/SMRT/Sin3A co-repressors and SMAD proteins. The interactions between Ski and these various complexes result in transcriptional repression of distinct signaling pathways, which are important for cell growth and proliferation (Jepsen and Rosenfeld, 2002; Nomura *et al.*, 1999). Deregulation of these pathways has also been observed in various human cancers. Ski expression is up regulated in many tumors types, such as melanomas, esophageal carcinomas, and leukemia (Fukuchi *et al.*, 2004; Kronenwett *et al.*, 2005; Pearson-White *et al.*, 1995; Reed *et al.*, 2001). In addition, Ski can cause highly malignant erythroleukemia (Larsen *et al.*, 1992), and transform hematopoietic multipotent progenitors (Dahl *et al.*, 1998a). In addition, increased expression of Ski can induce immortalization and self-renewal of primary multipotential myeloid progenitor cells from avian bone marrow (Beug *et al.*, 1995). This latter property of Ski is reminiscent of the effects of expressing a dominant-negative form of the retinoic acid receptor in hematopoietic cells (Tsai *et al.*, 1994), and thus may reflect the ability of Ski to repress retinoic acid signaling.

Retinoic acid (RA), a derivative of vitamin A, is essential for mammalian development. It functions by activating transcription involving the nuclear hormone receptor family of retinoic acid receptors (RARs). In the absence of ligand, retinoid receptors are primarily in the nucleus bound to RA responsive elements (RARE) as asymmetric, oriented RAR/RXR heterodimers. These complexes contain various co-repressors such as N-CoR and SMRT and repress transcription in the absence of ligand (Aranda and Pascual, 2001; Glass and Rosenfeld, 2000). Facilitated by cellular retinoic acid-binding protein II, the ligand (RA) binds to and induces conformational changes of RAR/RXR complex. This ligand-binding triggers a cascade of events, which favor the interactions between RAR and RXR, increasing their DNA affinity and causes co-repressors release, followed by co-activators recruitment, chromatin decompaction, and transcription initiation (Depoix *et al.*, 2001; Dilworth and Chambon, 2001; Orphanides and Reinberg, 2000; Rastinejad *et al.*, 2000). Tight regulation of the RA signaling pathway is essential for physiological responses. Thus, an understanding of all the mechanisms that affect RA signaling is important for our understanding of normal and abnormal physiological responses controlled by this signaling pathway.

More and more evidence shows Ski plays an important role in the regulation of transcription induced by RA. Initial analysis of mice genetically engineered to be null for the Ski gene revealed exencephaly, severe neural and muscle problems and the mice die at or just before birth (Berk *et al.*, 1997). More recently, the mice have defects in myelination, as well as craniofacial, digit and hematological defects (Atanasoski *et al.*, 2004; Colmenares *et al.*, 2002). Interestingly, some of the craniofacial defects are similar to birth defects associated with excessive intake of retinoids in humans. Detailed analysis

of the ocular defects in the mice has also indicated that they are similar to those associated with excessive intake of retinoid in humans and the authors claim that these defects are most consistent with a hypothesis that the Ski $^{-/-}$ mice is hypersensitive to retinoids (McGannon *et al.*, 2006). In humans, the Ski gene maps to chromosome 1p36.3. Terminal deletions of chromosome 1 in humans gives rise to monosomy 1p36 syndrome (Slavotinek *et al.*, 1999). Children with this syndrome have craniofacial defects and digit defects, which are virtually identical to those seen in the Ski$^{-/-}$ mice, imply that these defects may be due to reduced levels of Ski and reflect a hypersensitivity to retinoids (Colmenares *et al.*, 2002). Finally as mentioned above increased expression of Ski in myeloid precursors mimics the phenotype seen by expressing dominant-negative RAR. These data indicate that Ski may play a physiological role in regulating the repression of RA signaling during development.

Increased expression of Ski in hematopoietic cells in an animal model system gives rise to leukemia of progenitor cells of the myeloid and erythroid lineages. Furthermore, we determined that the increased expression of Ski repressed RA-induced transcription, which was important for the resistance to RA-induced differentiation and the subsequent development of leukemia (Dahl *et al.*, 1998a). Recently in collaboration with others we extended these studies and showed that Ski expression was increased by two-three fold in a subset of human acute myeloid leukemia, AML, patients and this increased expression correlated with bad prognosis and resistance to all trans-retinoic acid (ATRA)-induced differentiation therapy (Ritter *et al.*, 2006a). Although mechanistically distinct from the resistance to RA-induced differentiation seen in APL patients, these observations implicate the regulation of the RA pathway by Ski as being

important in this subset of AML patients and indicate that the role of Ski in leukemia development in animal models and humans involves repression of the RA signaling pathway. The specific mechanism by which Ski represses RA signaling is unknown.

Recent experiments have shown that stabilization of inactive Smad complexes on DNA is a critical event in c-Ski-mediated inhibition of TGF-β signaling(Suzuki *et al.*, 2004), In this chapter, we found that increased expression of Ski led to stabilization of proteins in the RA-repression complex, namely HDAC3 as well as RARα. These data indicate that like repression of TGF-β signaling by Ski, repression of RA signaling by Ski also involves stabilization of repression complexes and may indicate a common mechanism by which Ski is able to repress transcription of disparate signaling pathways.

B. Results

1. RA-induced RARα degradation was important for optimal RARα-mediated trans-activation.

RA can induce the degradation of its receptor RARα (Kopf *et al.*, 2000; Tanaka *et al.*, 2001). This degradation is thought to be a resetting mechanism for RAR-mediated transactivation and important for efficient RA target gene expression (Andela and Rosier, 2004). Therefore, we considered it possible that high-level expression of Ski might inhibit RA signaling by influencing this degradation. In order to investigate this possibility, we first demonstrated that in our hands RA did increase RARα turnover. We introduced Flag tagged RARα into cells and compared the turnover of RARα in the absence and presence of RA by Western blot analysis. We used cycloheximide (CHX) to stop new protein synthesis. As shown in Figure 2.1A, RA clearly increased the turnover of RARα. To see if this turnover was dependent on the ubiquitin proteasome pathway, we treated the transfected cells with proteasome pathway inhibitor MG132. As can be seen in Figure 2.1B, the addition of MG132 inhibited the turnover of RARα indicating that this degradation involved the ubiquitin proteasome pathway. To determine if the turnover of RARα had any affects on the RA signaling, we performed a luciferase reporter assay using a CRBPII-Luc reporter. First, we checked the RA-depended luciferase activity by transiently transfected cells with a CRBPII-Luc reporter construct which contained RA response element upstream of the luciferase reporter gene, plasmids encoding Flag-RARα and RXR for 24 hrs and treated the cells with RA for different time, then checked the activity of the luciferase reporter gene. From the data (Figure 2.2A), we can see that increasing RA treatment time led to increases in the RA

dependent luciferase activity. However, the Flag-RARα protein level decreased with increasing RA treatment time. Next, we treated cells with RA for 12 hrs and proteasome inhibitor, MG132, for 5 hrs or 12 hrs. We then did reporter assay to check the activity of the reporter gene. As can be seen in Figure 2.2B, the addition of RA resulted in an about 4-fold increase of luciferase activity. The addition of 5μM MG132 inhibited this induction in a time-dependent manner. Western blot analysis showed proteasome inhibitor MG132 treatment inhibited Flag-RARα degradation, and at the same time, the RA-dependent transcriptional activity decreased. β-actin was used as a loading control for the Western blot analysis. To rule out the possibility that the effects of MG132 were due to non-specific toxicity to the cells, we determined the effects of 5μM MG132 on a TK-Luc reporter. 5μM MG132 had no effect on this reporter gene trans-activation as shown in Figure 2.2C, indicating that the MG132 effect on CRBPII-Luc reporter gene expression was to some extent RA specific. These data suggested that, as reported by others (Andela and Rosier, 2004; Kopf *et al.*, 2000; Tanaka *et al.*, 2001), RA could induce RARα degradation, and this degradation was important for optimal RA signaling.

2. Ski expression inhibited RA-induced RARα degradation

Having established that we could measure the effect of RA on RARα turnover, we next determined the effects of expressing Ski on this turnover rate. In order to exclude the possibility that Ski might also have some effects on transcription or translation of RARα, we used CHX to inhibit new protein synthesize after 24 hrs transfection. Figure 2.3A showed that the expression of Ski slowed down the RA-induced turnover of RARα. We quantified this effect as shown in Figure 2.3B, the expression of Ski clearly slowed down the rate of RA-induced RARα degradation. Since, as shown above, the

degradation of RARα involved the proteasome, we also determined the effect of Ski expression on RARα ubiquitination by using an in vivo ubiquitination assay. As can be seen in Figure 2.4, there was a marked reduction of ubiquitinated forms of RARα, indicating that the ubiquitination and subsequent ubiquitin-dependent degradation of RARα was inhibited by the presence of Ski.

3. Ski and RARα were in the same complex in both the presence and absence of RA

Ski interacts with RARα (Dahl *et al.*, 1998a; Ritter *et al.*, 2006b). Therefore, we looked at the interaction of RARα and Ski in the absence and presence of RA. We could show Ski and RARα interaction by co-immunoprecipitation and the level of this interaction was unaffected by 6 hrs RA treatment (Figure 2.5A). Similarly, immunofluorescence studies showed that Ski and RARα were both present in the nuclear dot structures (Figure 2.5B), previously shown as the site of intranuclear localization for Ski (Sutrav*e et al.*, 1990a), and this co-localization was unaffected by the addition of RA. The above data indicated Ski was somewhat different from other co-repressors in that it was in the same complex with RARα in both the absence and presence of RA treatment**.**

4. Ski associated with HDAC3 and inhibited HDAC3 degradation

Ski can act as a co-repressor, and has been shown to interact with other co-repressors, N-CoR/SMRT and mSin3A (Nomura *et al.*, 1999). The nuclear receptor co-repressor complex, which is necessary for the nuclear receptor-mediated repression, consists of N-CoR,/SMRT, HDAC3, transducin β-like 1 (TBL1), TBLR1 and GPS2 (Perissi *et al.*, 2004) and probably several more proteins (Tabata *et al.*, 2009). This complex is important for the unliganded RAR-mediated repression and the degradation of

the co-repressor complex by the proteasome system is essential for efficient RA signaling (Andela and Rosier, 2004). The active degradation of the co-repressor proteins by the proteasome system is necessary in order for the recruitment of co-activator proteins to the RAR/RXR complex, and this is the co-repressor/co-activator exchange model of RA signaling (Perissi *et al.*, 2004). Since we showed above that Ski can inhibit RA-induced RARα degradation, we hypothesized that Ski might associate with the co-repressor complex bound to RARα and inhibit the degradation of proteins in this complex. A key functional member of this complex is histone deacetylase HDAC3, which is responsible for deacetylating histones and maintaining a close chromatin conformation. Thus, we wanted to determine if Ski could interact with HDAC3 and influence its turnover. As shown in Figure 2.6A, co-immunoprecipitation experiments demonstrated a Ski-HDAC3 interaction in both the absence and presence of RA. We next examined whether RA can induce HDAC3 degradation as would be predicted by the co-repressor/co-activator exchange model of RA signaling. In Figure 2.6B, we can see that RA did induce HDAC3 degradation and furthermore, MG132 inhibited this degradation. Having determined that we could monitor RA-induced HDAC3 degradation, we assessed the effect of expressing Ski on this turnover. As can be seen in Figure 2.6C, we found that Ski expression could inhibit HDAC3 turnover. Thus, these data indicated that the expression of Ski could result in the stabilization of HDAC3.

5. The inhibitory effects of Ski on RARα and HDAC3 were N-CoR independent

HDAC3 is in the complex with N-CoR (Perissi *et al.*, 2004) and Ski can interact with N-CoR (Nomura *et al.*, 1999; Ueki and Hayman, 2003b) . Therefore, it is possible that the interaction between Ski and HDAC3 is indirect and mediated by N-CoR. To

address this possibility, we made a point mutant of Ski, Flag-Ski (L127P), which was the equivalent point mutant of chicken Ski L110P that could not bind to N-CoR. First, we performed co-immunoprecipitation assay to check whether Flag-Ski (L127P) and N-CoR were still in the same complex. We used anti-Ski antibody to pull down wild type Flag-Ski (as a control) or Flag-Ski (L127P), and performed Western blot using anti-Flag antibody. As shown in Figure 2.7A, wild type Flag-Ski and N-CoR were in the same complex, and as expected, Flag-Ski (L127) no longer interacted with N-CoR. We then looked at the ability of this mutant to affect the turnover of HDAC3 and RARα. As can be seen in Figure 2.7B and 2.7C, expression of this point mutant of Ski still inhibited the turnover of RARα and HDAC3. These results showed that the stabilization effects of Ski on RARα and HDAC3 were N-CoR binding independent.

6. Ski can also inhibited endogenous HDAC3 turnovers

The above experiments used exogenously expressed proteins. For further investigation, we examined the effects of increased levels of Ski expression on endogenous HDAC3 levels. To do this we utilized mouse embryonic fibroblasts (MEF) isolated from Ski $^{-/-}$ mice. We introduced a Ski expressing retrovirus into these cells in which Ski expression was driven by a Doxycycline-regulated promoter. As shown in Figure 2.8A, in the Ski negative background of these MEF, we turned on Ski expression by the withdrawal of Doxycycline, whereas the addition of Doxycycline turned off Ski expression. Using this regulated expression of Ski, we examined the effects of Ski expression on the endogenous levels of HDAC3. As can be seen in Figure 2.8B, expression of Ski clearly inhibited RA-induced turnover of endogenous HDAC3. To complement these studies, we used the osteosarcoma cell line MG63 cells, which

expressed endogenous Ski. These cells were infected with a retrovirus expressing the short hairpin type RNA (shRNA) directed against Ski in order to knockdown the expression of Ski. Figure 2.8C showed that the shRNA did reduce the levels of Ski expression and this reduction of the endogenous Ski protein level correlated with reduced levels of HDAC3. Cells expressing control shRNA, lane 1 & 3, maintained Ski expression and increased levels of HDAC3. These data suggested that Ski could stabilize not only exogenously expressed HDAC3 but also endogenous HDAC3 and implied that expression of Ski was capable of stabilizing the co-repressor complex and by doing so Ski could repress transcription induced by RA.

C. Discussion

In 1981, all-trans retinoic acid, ATRA was reported to induce terminal differentiation of human promyelocytic cells in culture (Breitman *et al.*, 1981), and it was subsequently shown that ATRA could induce clinical remissions in acute promyelocytic leukemia (APL) (Huang *et al.*, 1987). APL contains a chromosomal translocation that involves the RAR gene, which is in part explains the sensitivity of this specific leukemia to ATRA. Since that time, ATRA has been widely used clinically for the treatment of APL patients. Recently, Ski expression has been shown to be up-regulated by two- three fold in a subset of human acute myeloid leukemia, AML, and this increased expression correlates with bad prognosis and resistance to ATRA-induced differentiation therapy (Ritter *et al.*, 2006a), implying that repression of RA signaling by Ski may be playing a role in this subset of AML patients. In addition, several reports in other cell types and system suggest that Ski may play a physiological role in regulating the repression of RA signaling (Atanasoski *et al.*, 2004; Berk *et al.*, 1997; Colmenares *et al.*, 2002; McGannon *et al.*, 2006; Slavotinek *et al.*, 1999). However, the molecular mechanism of how Ski can repress RA signaling is still unclear. In this chapter, we have examined the effects of Ski expression on the stability of two key members of the RA co-repressor complex and found that Ski expression stabilized these proteins.

This stabilization may be responsible for the ability of Ski to repress RA signaling. Previous reports have indicated that nuclear hormone-mediated nuclear hormone receptor (NR) degradation is necessary for efficient target genes activation. This has been reported for several NRs, including estrogen receptor α (ERα) (Lonard *et al.*, 2000), progesterone receptor (PR) (Lange *et al.*, 2000), androgen receptor (AR) (Lin *et al.*, 2002), thyroid

hormone receptor (TR) (Dace *et al.*, 2000), and retinoic acid receptor α (RARα) (Zhu *et al.*, 1999). Furthermore, it has also been reported that co--repressor/co-activator complex exchange is required for transcriptional activation by RARα and other regulated transcription factors (Perissi *et al.*, 2004). In this study, we found that Ski could inhibit RA-induced RARα degradation and stabilize HDAC3. These data are consistent with the hypothesis that the presence of Ski inhibits co-repressor/co-activator complex exchange and thus results in inhibition of RA signaling.

The nuclear receptor co-repressor complex, which is necessary for the nuclear receptor-mediated repression, consists of N-CoR,/SMRT, HDAC3, transducin β-like 1 (TBL1), TBLR1 and GPS2 (Perissi *et al.*, 2004) and probably several more proteins (Tabata *et al.*, 2009). We tested whether Ski can also regulate the protein level of HDAC3, which could provide enzymatic activity and promote transcription repression by deacetylating histones (Sengupta and Seto, 2004). Our data showed that Ski and HDAC3 associated with each other in both the absence and presence of RA (Figure 2.6A). Consistent with model proposed by Perissi *et al.*, who reported RA-dependent active degradation of co-repressor complex, we found that RA indeed also induced HDAC3 proteasome degradation (Figure 2.6B) (Perissi *et al.*, 2004). Similar to the effects on RA-induced RARα degradation, Ski could also inhibit RA-mediated HDAC3 proteasome turnover (Figure 2.6C).

N-CoR, a known binding partner of Ski, is important for Ski-mediated repression of nuclear receptor signaling (Ueki and Hayman, 2003b), the previous data from our lab also indicated the Ski repressive effect on RARα signaling was N-CoR dependent (Ritter *et al.*, 2006a). We found that the Ski point mutant Ski (L127P), which could not bind to

N-CoR, was still able to stabilize RARα and HDAC3 (Figure 2.7), indicating the stabilization effects of Ski was N-CoR independent. Even though Ski (L127P) point mutant still stabilized RARα and HDAC3, our luciferase assay indicated it could not repress RA signaling pathway, which might imply that stabilization effects of Ski on RARα and HDAC3 was necessary but not sufficient for RA signaling repression. The N-CoR binding ability of Ski somehow contributed to the intact function of Ski.

In order to test the effects of Ski on endogenous RARα and HDAC3 levels, we generated MEF $Ski^{-/-}$ cells, into which we introduced a Ski gene under the control of Doxycycline (Figure 2.8A). We demonstrated that the regulated Ski expression could also stabilize endogenous levels of HDAC3 (Figure 2.8B). To complement this analysis, we used shRNA directed against Ski to reduce the levels of endogenous Ski protein in MG63 osteosarcoma cell line. As predicted, reduction in the level of expression of Ski led to a parallel reduction of HDAC3 levels. These data together indicated that Ski levels could influence the levels of HDAC3 expression.

Ski has been shown recently to stabilize the inactive Smad complex on the Smad binding element (SBE) of TGF-β target genes, and this stabilization inhibits the access of newly activated Smad proteins to the SBE (Suzuki *et al.*, 2004). Thus, the stabilization of the inactive complex by Ski results in the inhibition of TGFβ signaling. In a mechanistically similar manner, we reported here that Ski might maintain the repressed state of the RA target genes by stabilizing RARα as well as the HDAC3 containing co-repressor complex. Our data suggested that Ski could stabilize RARα by inhibiting RA-induced RARα proteasome degradation (Figure 2.3). It is well known that in the presence of ligand treatment, the co-repressors are dissociated from the receptors and the

coactivators are recruited for the following transcription activation (Depoix *et al.*, 2001; Dilworth and Chambon, 2001; Orphanides and Reinberg, 2000; Rastinejad *et al.*, 2000). Thus, the stabilization of HDAC3 by Ski would result in an inhibition of this exchange and, as in the case of the SMAD proteins, result in repression of transcription.

C-Ski is the founding member of a family of proteins that includes itself and Ski-related protein Sno. The Ski-related protein SnoN can positively regulate the protein levels of the co-repressor mSin3A. Wilkinson *et al*. found that mSin3A protein levels dramatically reduced in Hepa 1-6 cells in which the levels of SnoN decreased. Furthermore, they also found that the degradation of SnoN and Sin3A after TGF-β treatment occurred in parallel as if the levels of mSin3A were linked to the level of SnoN (Wilkinson *et al.*, 2008). These data indicate that like Ski, its family member SnoN can also regulate the levels of co-repressors, mSin3A. These findings together with ours point to similar mechanistic functions of these family members by stabilizing the expression of co-repressors to repress transcription.

Based on our data, we hypothesize that the mechanism of Ski's repressive effects on RA signaling is primarily at the level of co-repressor/co-activator exchange by stabilizing proteins in the co-repressor complex in the presence of ligand. Figure 2.9 shows a simplified model of co-repressor/co-activator exchange, as proposed by Perissi *et al.*, and modified to incorporate a role for Ski. As depicted in the model, RARα and RXR form a heterodimer and bind to the RA responsive elements (RARE), which is upstream of RA target genes. In the absence of ligand, RARα/RXR can recruit N-CoR/SMRT, HDAC3 and several other cofactors, and form a co-repressor complex to repress transcription. In the presence of ligand (RA), RA can bind to and induce conformational

changes of RARα/RXR complex, which causes co-repressors release and degradation through ubiquitin proteasome pathway, and co-activators recruitment, chromatin decompaction, and transcription initiation. When Ski protein is present at high levels it is capable of associating with the co-repressor complex by virtue of its ability to interact, directly or indirectly, with RARα, N-CoR/SMRT and HDAC3, and in doing so, Ski prevents the efficient dissociation and/or degradation of the co-repressor complex. Since the degradation of the co-repressor complex is inhibited, the co-activator complex cannot be recruited and target genes expression is inhibited even in the presence of RA. This inhibition of co-repressors degradation means that efficient transcription does not occur and since RARα degradation takes places after the transcription of the target genes, the inhibition of RARα degradation might be indirectly. Thus, we hypothesize that the primary action mode of Ski is to stabilize the co-repressor complex and this indirectly results in the stabilization of RARα

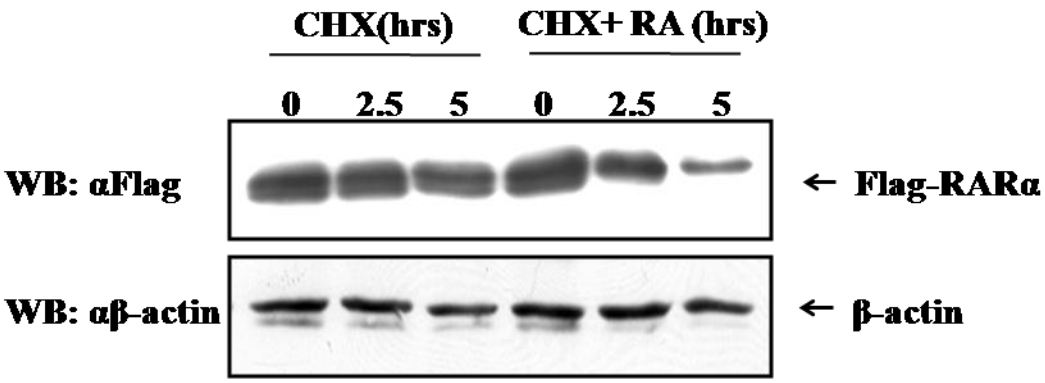
Ski is capable of modulating the action of many signaling pathways driven by diverse transcription factors, such as SMADs, AP-1, Vitamin D receptor (Akiyoshi *et al.*, 1999; Luo *et al.*, 1999; Ueki and Hayman, 2003b; Xu *et al.*, 2000). It is possible that the ability of Ski to inhibit transcription driven by these distinct transcription factors will also involve the stabilization of co-repressor complexes. In fact, as discussed above, stabilization of SMAD complexes on DNA by Ski has already been reported (Suzuki *et al.*, 2004). Thus, this ability of Ski to stabilize repression complexes may reflect the underlying mechanism by which Ski can regulate signaling mediated by such diverse groups of transcription factors.

In summary, we propose that Ski protein inhibits the RA signaling pathway through maintaining the basal repressed state of the RA target genes by stabilizing the co-repressor complex and RARα. Our findings reveal a novel mechanism of Ski's repressive effect on RA signaling pathway and this mechanism will apply to other signaling pathways inhibited by the Ski protein.

Figure 2.1 RA-mediated RARα proteasome degradation

A. COS-1 cells, transfected with plasmid encoding Flag-RARα, were treated without or with 1μM ATRA for different times in the presence of CHX as indicated. Flag-RARα levels were detected by Western blot analysis with an anti-Flag antibody, and the β-actin level was used as a loading control.
B. COS-1 cells were transfected with plasmid encoding Flag-RARα. After 24 hrs transfection, cells were treated without or with ATRA for 12 hrs in the absence or presence of 1μM MG132 as indicated. Flag-RARα levels were detected with an anti-Flag antibody, the β-actin level was used as a loading control.

A


CHX(hrs)
CHX+ RA (hrs)
0 2.5 5 0 2.5 5
WB: αFlag
← Flag-RARα
WB: αβ-actin
← β-actin

B

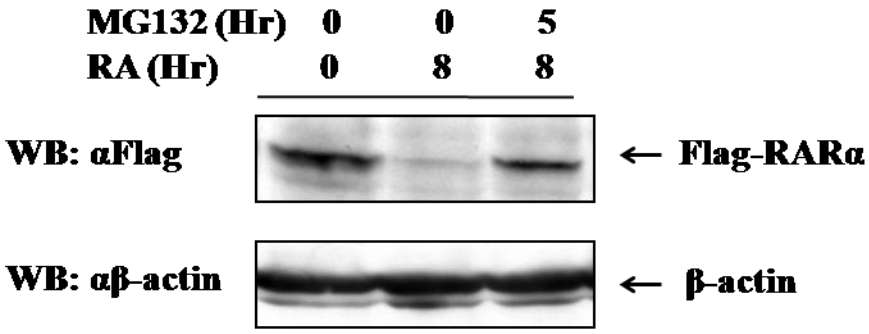

MG132 (Hr) 0 0 5
RA (Hr) 0 8 8
WB: αFlag
← Flag-RARα
WB: αβ-actin
← β-actin

Figure 2.2 RA-mediated RARα degradation was required for the activation of RA signaling

A. QT6 cells were transfected with CRBPII-Luc, Flag-RARα and RXR, and untreated or treated with RA (1μM) for different time after 24 hrs transfection. Western blot analysis showed the Flag-RARα levels under different conditions and β-actin level was used as a loading control.
B. QT6 cells were transfected with CRBPII-Luc, Flag-RARα and RXR, and untreated or treated with RA (1μM) for 12 hrs after 24 hrs transfection. MG132 (5μM) treatment was as indicated. Western blot analysis showed the Flag-RARα levels under different conditions and β-actin level was used as a loading control.
C. QT6 cells were transfected with TK-luc, and treated with or without MG132 (5μM) for 12 hrs after 24 hrs transfection.
   The results were expressed as means ± S.D. from three independent experiments. *RLU*, relative light units (arbitrary activity)

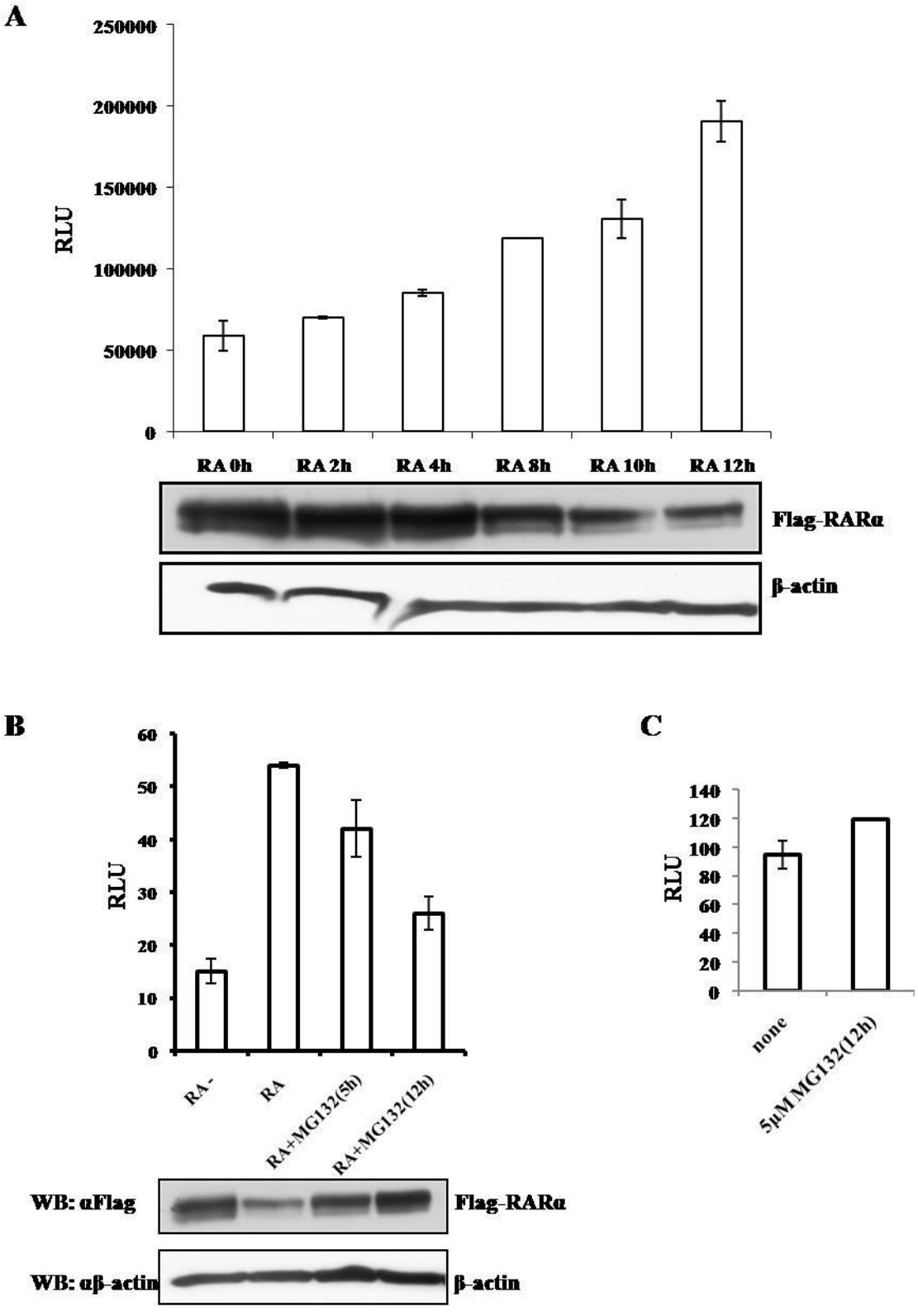
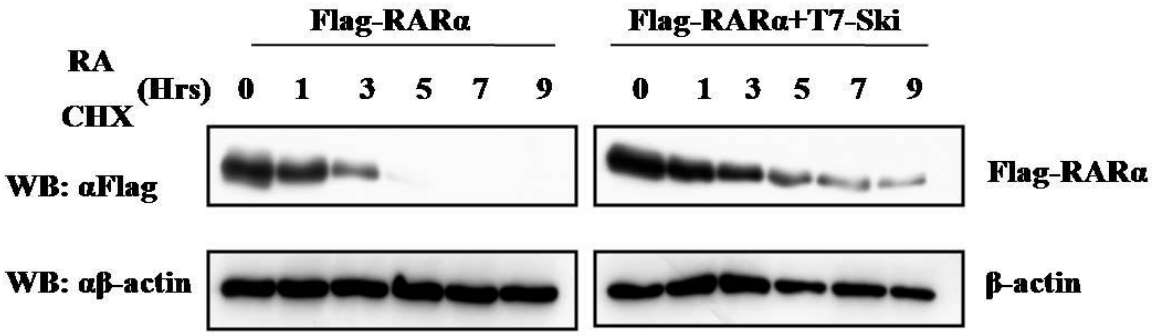

A
250000
200000
150000
100000
50000
0
RLU
RA 0h
RA 2h
RA 4h
RA 8h
RA 10h
RA 12h
Flag-RARα
β-actin
B
60
50
40
30
20
10
0
RLU
RA-
RA
RA+MG132(5h)
RA+MG132(12h)
WB: αFlag
Flag-RARα
WB: αβ-actin
β-actin
C
140
120
100
80
60
40
20
0
RLU
none
5μM MG132(12h)

Figure 2.3 Ski inhibited RA-induced RARα degradation

A. We transfected COS-1 cells with Flag-RARα, with or without T7-Ski, and then added CHX and RA to the cells after 24 hrs transfection. The cell lysates were collected at different time points: 0 h, 1 h, 3 hrs, 5 hrs, 7 hrs, 9 hrs. Flag-RARα levels were checked by Western blot analysis using an anti-Flag antibody, the β-actin level was used as a loading control.
B. The quantification of results from A The band intensities were quantified with Odyssey software (LI-COR Biosciences).

A


Flag-RARα
Flag-RARα+T7-Ski
RA
CHX
(Hrs) 0 1 3 5 7 9 0 1 3 5 7 9
WB: αFlag
Flag-RARα
WB: αβ-actin
β-actin

B

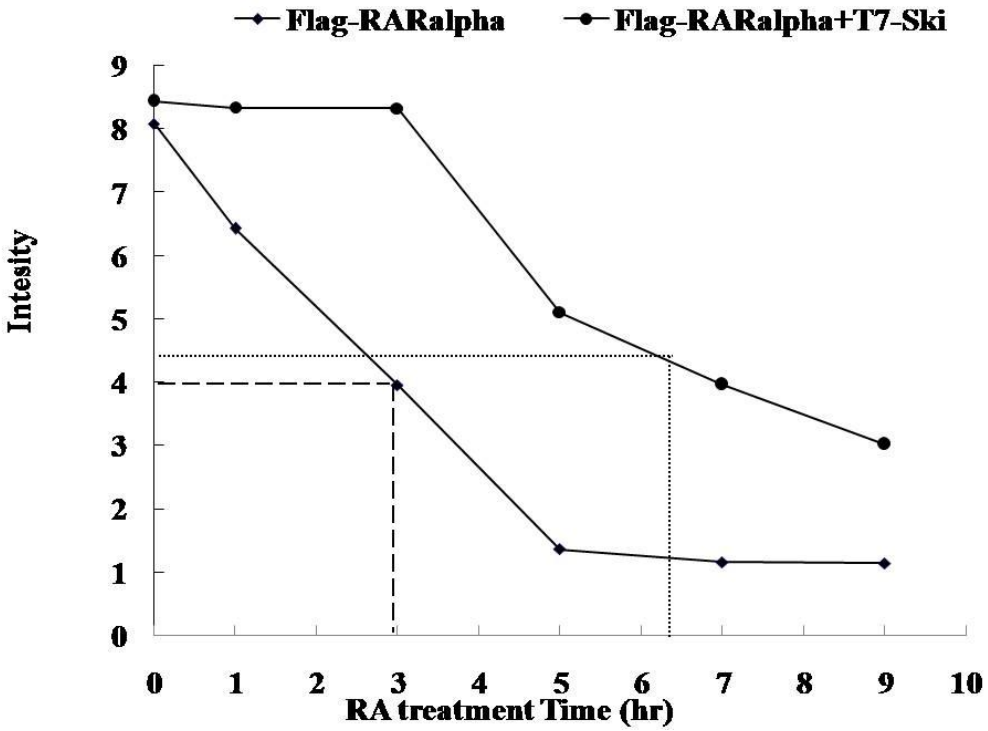

Flag-RARalpha
Flag-RARalpha+T7-Ski
Intesity
0 1 2 3 4 5 6 7 8 9
0 1 2 3 4 5 6 7 8 9 10
RA treatment Time (hr)

Figure 2.4 Ski inhibited RA-induced RARα proteasome ubiquitination

QT6 cells were transfected with different combinations of three plasmids: Flag-RARα, T7-Ski and 6His-Ub as indicated for 24 hrs, and then treated with RA for 2 hrs. The ubiquitinated forms of Flag-RARα were purified using Ni-NTA- agarose beads, and detected using an anti-Flag antibody by Western blot analysis. The upper panel showed the ubiquitinated Flag-RARα, which were purified using Ni-NTA- agarose, the lower panel showed the Flag-RARα level in the cell lysates.

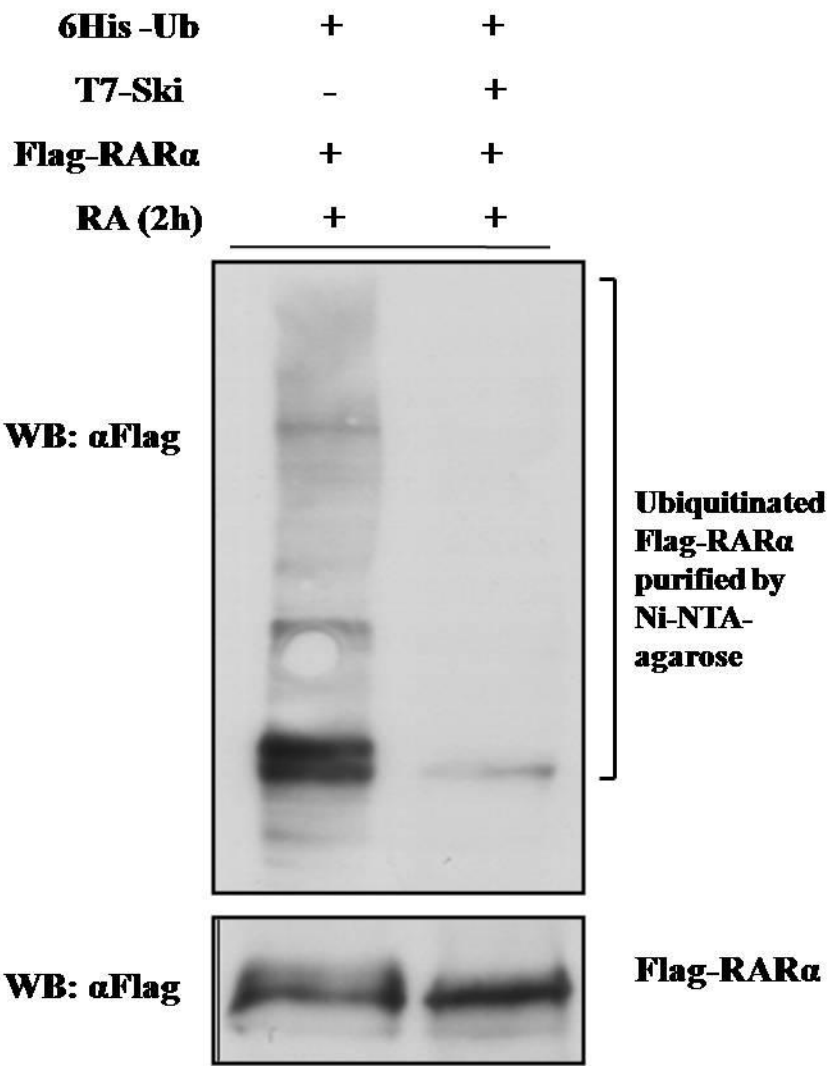

6His -Ub + +
T7-Ski - +
Flag-RARα + +
RA (2h) + +
WB: αFlag
Ubiquitinated Flag-RARα purified by Ni-NTA-agarose
WB: αFlag
Flag-RARα

Figure 2.5 Ski and RARα were in the same complex in both the presence and absence of RA treatment

A. Co-immunoprecipitation in COS-1 cells showed Ski and RARα interaction in both the presence and absence of RA treatment. Ski was co-immunoprecipitated with Flag-RARα using an anti-Flag antibody, and a mouse normal IgG as a control. The immunocomplexes and 10% input were analyzed by Western blot analysis using an anti-T7 antibody (the upper panel) or an anti-Flag antibody (the lower panel).
B. Shown were representative fluorescent images of HeLa cells expressing Myc-RARα and GFP-Ski without (A-C) or with (D-F) 6 hrs RA (1μM) treatment. Myc-RARα were detected by an anti-RARα (rabbit) antibody, Alexa Fluor 546 goat anti-rabbit was used as a secondary antibody and showed red under the microscope. GFP-Ski showed green. Hoechst was used for DNA staining, which was blue.

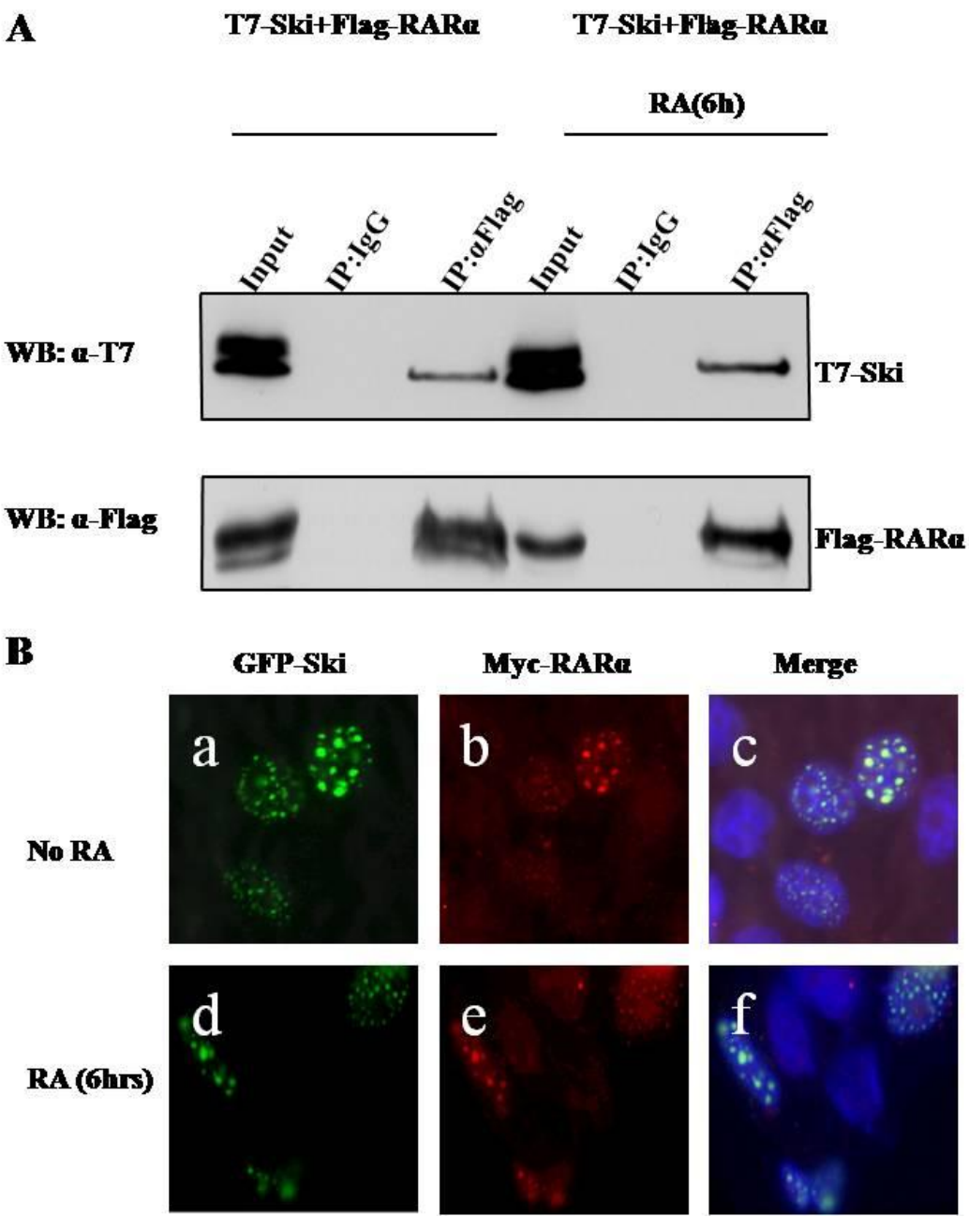

A
T7-Ski+Flag-RARα
T7-Ski+Flag-RARα
RA(6h)
Input
IP:IgG
IP:αFlag
Input
IP:IgG
IP:αFlag
WB: α-T7
T7-Ski
WB: α-Flag
Flag-RARα
B
GFP-Ski
Myc-RARα
Merge
No RA
RA (6hrs)
a
b
c
d
e
f

Figure 2.6 Ski associated with the HDAC3 and inhibited HDAC3 degradation

A. Co-immunoprecipitation in COS-1 cells showed Ski and HDAC3 interaction in both the presence and absence of 6 hrs RA treatment. Flag-HDAC3 was co-immunoprecipitated with Flag-Ski using an anti-Ski antibody (rabbit), and a rabbit normal IgG as a control. The immunocomplexes and 10% input were detected by an anti-Flag antibody.
B. COS-1 cells were transfected with plasmid encoding Flag-HDAC3. After 24 hrs transfection, cells were treated without or with ATRA for 18 hrs in the absence or presence of 5μM MG132 as indicated. Flag-HDAC3 levels were detected by Western blot analysis with an anti-Flag antibody, the α-tubulin level was used as a loading control.
C. We transfected COS-1 cells with Flag-HDAC3 with or without T7-Ski, and then added CHX and RA to the cells after 24 hrs transfection. The cell lysates were collected at different time points: 0 h, 4 hrs, 8 hrs. Flag-HDAC3 levels were checked by Western blot using an anti-Flag antibody (the middle panel), Ski levels were determined by anti-T7 antibody (the upper panel) and the α-tubulin level was used as a loading control (the lower panel).

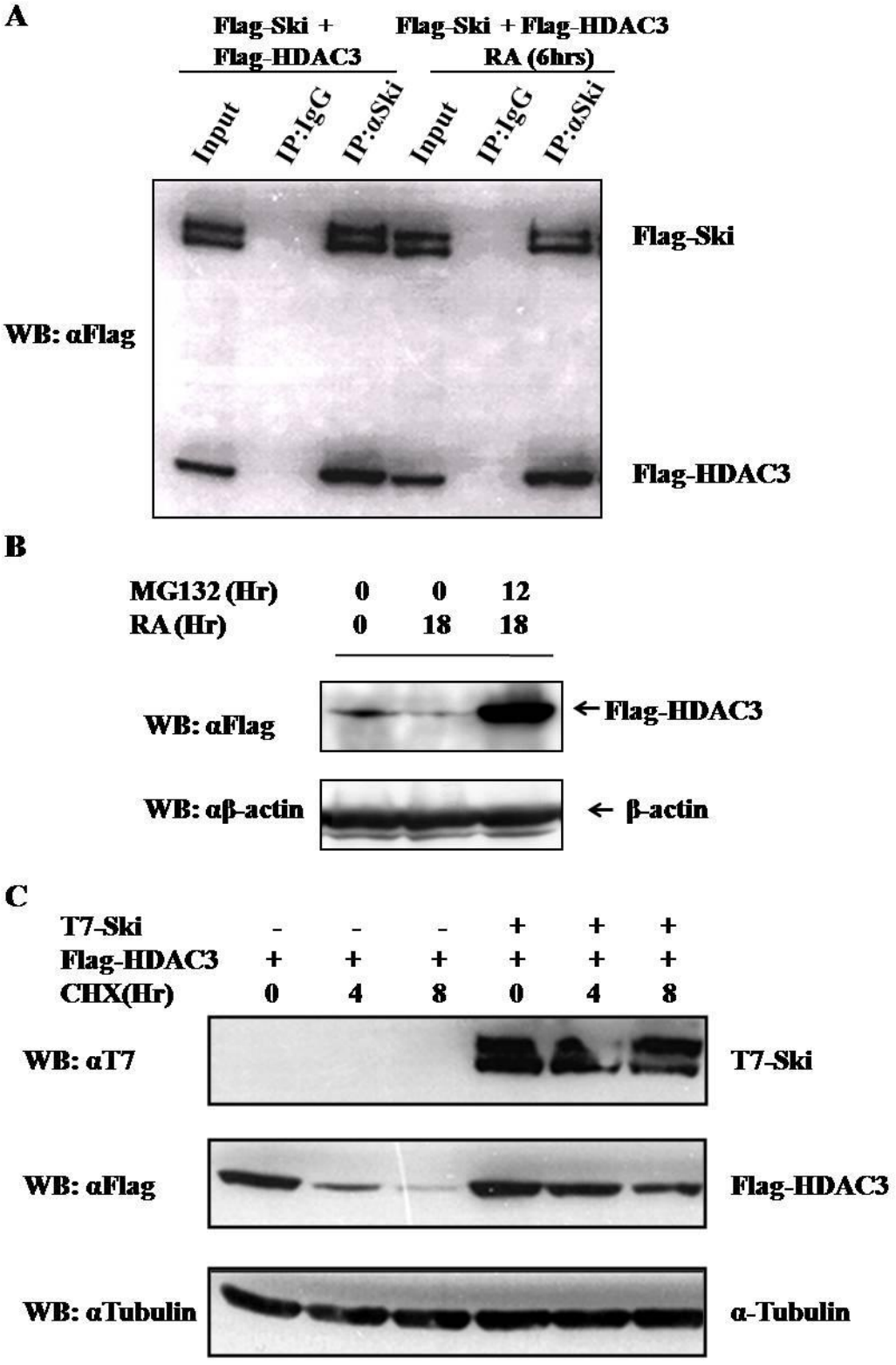

A
Flag-Ski + Flag-HDAC3
Flag-Ski + Flag-HDAC3 RA (6hrs)
Input
IP:IgG
IP:αSki
Input
IP:IgG
IP:αSki
WB: αFlag
Flag-Ski
Flag-HDAC3
B
MG132 (Hr) 0 0 12
RA (Hr) 0 18 18
WB: αFlag
← Flag-HDAC3
WB: αβ-actin
← β-actin
C
T7-Ski - - - + + +
Flag-HDAC3 + + + + + +
CHX(Hr) 0 4 8 0 4 8
WB: αT7
T7-Ski
WB: αFlag
Flag-HDAC3
WB: αTubulin
α-Tubulin

Figure 2.7 The stabilization effects of Ski on HDAC3 and RARα were independent on its N-CoR binding activity.

A. Wt- Flag-Ski and Flag-Ski (L127P) were co-immunoprecipitated with Flag-N-CoR-C using an anti-Ski antibody (rabbit), and a rabbit normal IgG was used as a control. The immunocomplexes and 10% input were detected by Western blot using an anti-Flag antibody
B. COS-1 cells were transfected with Flag-RARα with or without Flag-Ski (L127P), and then CHX and RA were added to the cells after 24 hrs transfection. The cell lysates were collected at different time points: 0 h, 2 hrs, and 4 hrs. Flag-RARα and Flag-Ski (L127P) levels were checked by an anti-Flag antibody, and the α-tubulin level was used as a loading control.
C. COS-1 cells were transfected with Flag-HDAC3 with or without Flag-Ski (L127P), and CHX and RA were added to the cells after 24 hrs transfection. The cell lysates were collected at different time points: 0 h, 3 hrs, and 7 hrs. Flag-HDAC3 and Flag-Ski (L127P) levels were checked by using an anti-Flag antibody, and the α-tubulin level was used as a loading control.

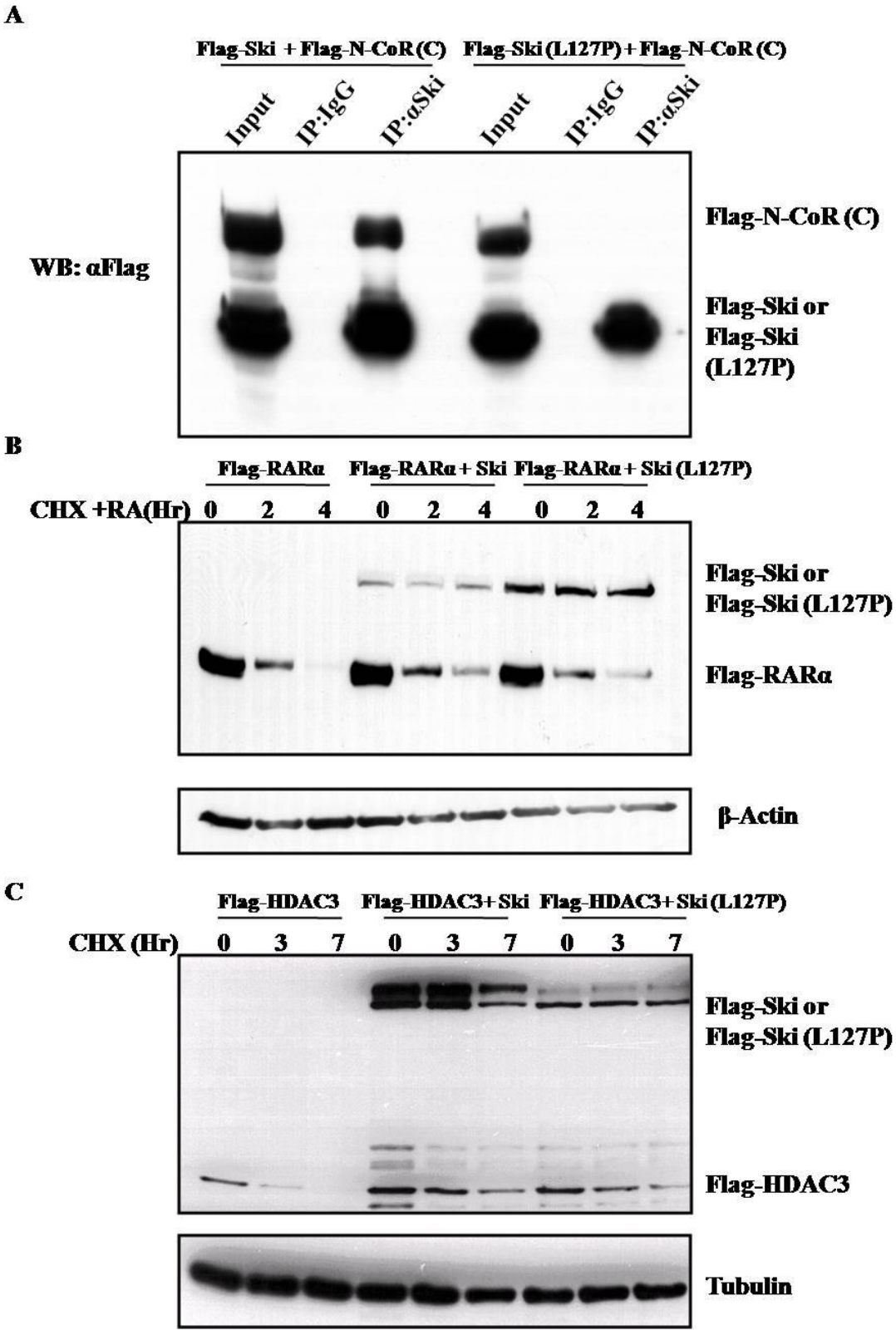

A
Flag-Ski + Flag-N-CoR (C)
Flag-Ski (L127P) + Flag-N-CoR (C)
Input
IP:IgG
IP:αSki
Input
IP:IgG
IP:αSki
WB: αFlag
Flag-N-CoR (C)
Flag-Ski or
Flag-Ski
(L127P)
B
Flag-RARα
Flag-RARα + Ski
Flag-RARα + Ski (L127P)
CHX +RA(Hr)
0 2 4 0 2 4 0 2 4
Flag-Ski or
Flag-Ski (L127P)
Flag-RARα
β-Actin
C
Flag-HDAC3
Flag-HDAC3+Ski
Flag-HDAC3+Ski (L127P)
CHX (Hr)
0 3 7 0 3 7 0 3 7
Flag-Ski or
Flag-Ski (L127P)
Flag-HDAC3
Tubulin

Figure 2.8 Ski could inhibit endogenous HDAC3 degradation.

A. MEF $Ski^{-/-}$ cells, which have a Ski gene under the control of Doxycycline, were maintained in the absence (lane2) and presence (lane1 and lane3) of 2μg/ml Doxycycline for 48 hrs, and the Ski expression was detected by an anti-Ski antibody (G8).
B. The MEF $Ski^{-/-}$ cells were maintained in the presence or absence of Doxycycline for 48 hrs, then untreated or treated with CHX as indicated. The endogenous HDAC3 levels were determined by an anti-HDAC3 antibody.
C. The osteosarcoma cell line MG63 cells were infected with a retrovirus expressing the short hairpin-type RNA (shRNA) directed against Ski in order to knockdown the expression of Ski (lane 2) and the endogenous HDAC3 levels were detected by an anti-HDAC3 antibody.

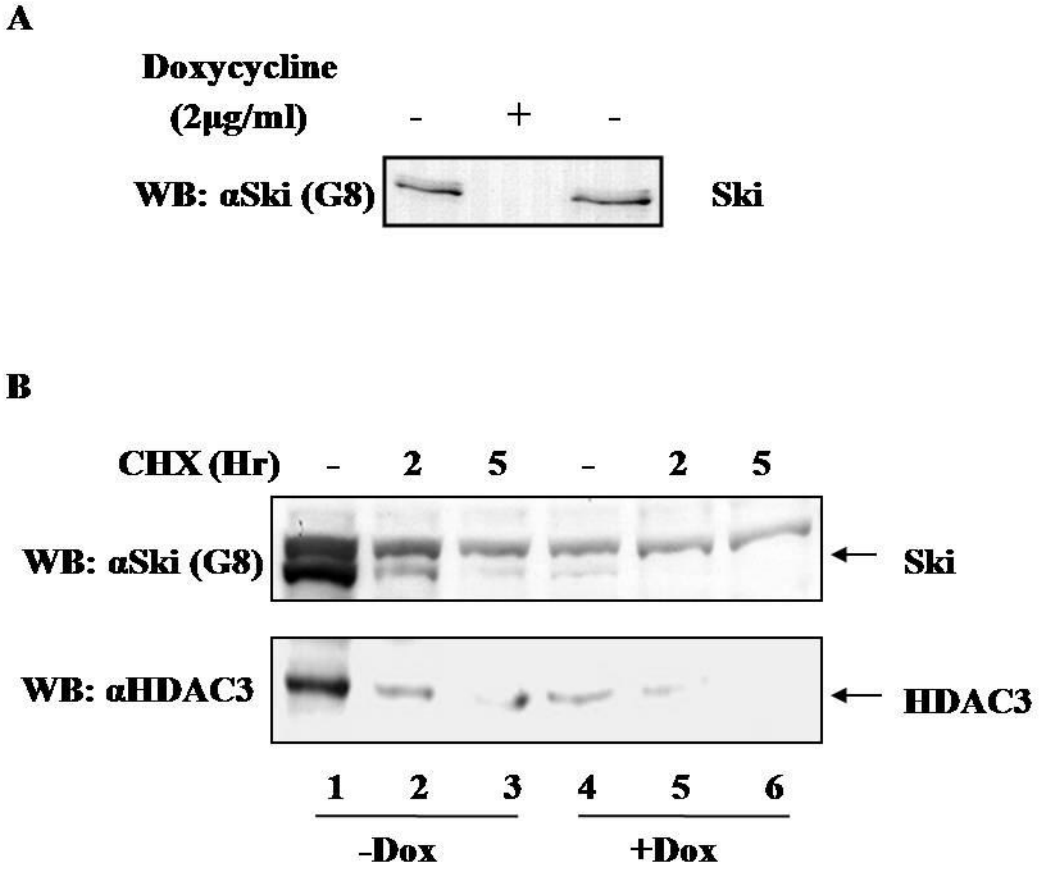
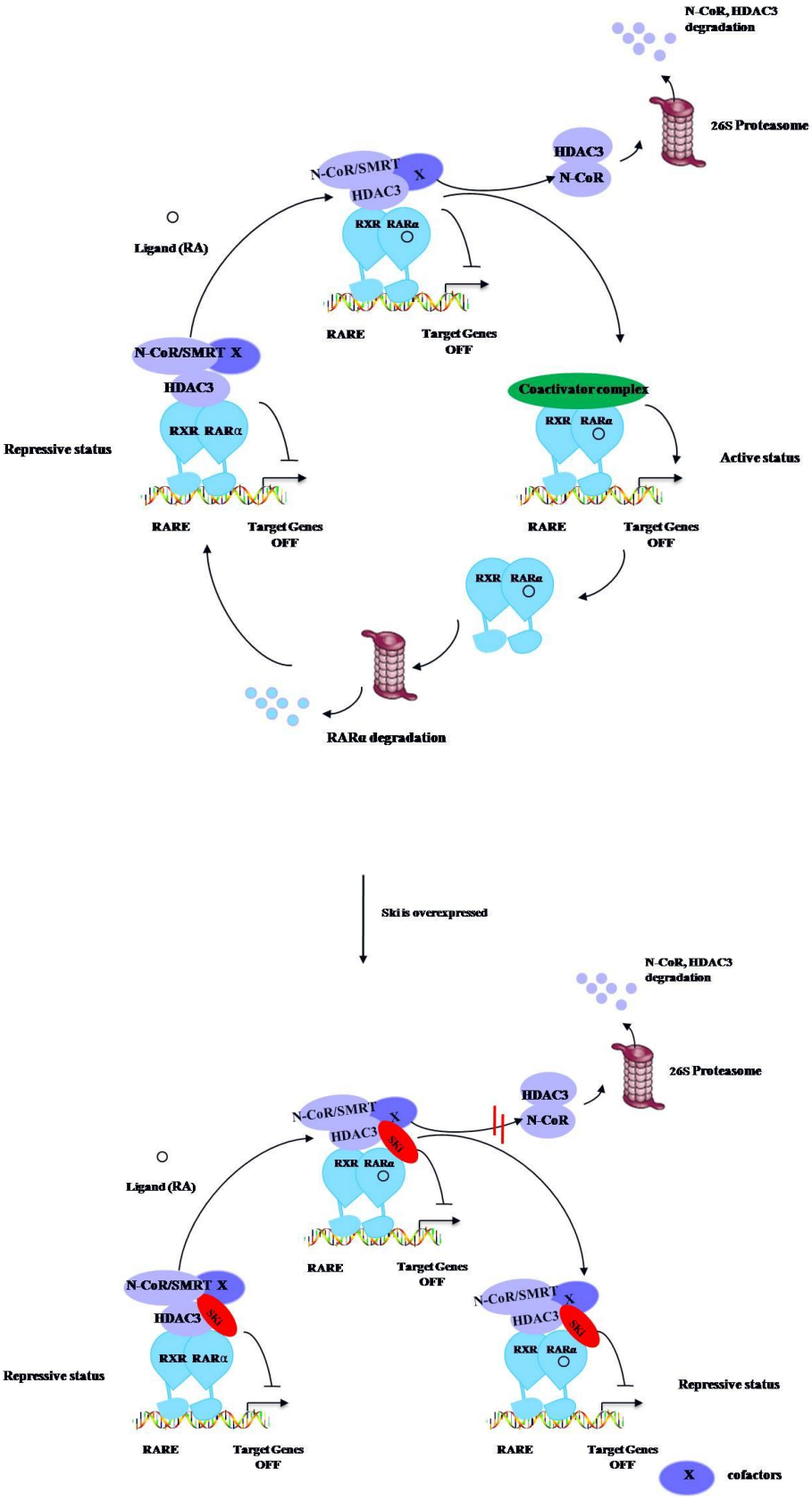

A
Doxycycline
(2μg/ml)
-
+
-
WB: αSki (G8)
Ski
B
CHX (Hr)
-
2
5
-
2
5
WB: αSki (G8)
Ski
WB: αHDAC3
HDAC3
1
2
3
4
5
6
-Dox
+Dox
C
shRNA
control
Ski
control
WB: αSki (G8)
Ski
WB: αHDAC3
HDAC3
1
2
3

Figure 2.9: Model for the mechanism of Ski-mediated repression of RA signaling. See text for details (based on the model proposed in (Perissi *et al.*, 2004)).


N-CoR, HDAC3 degradation
26S Proteasome
HDAC3
N-CoR
N-CoR/SMRT
X
HDAC3
RXR RARα
Ligand (RA)
RARE
Target Genes OFF
N-CoR/SMRT X
HDAC3
RXR RARα
Repressive status
RARE
Target Genes OFF
Coactivator complex
RXR RARα
Active status
RARE
Target Genes OFF
RXR RARα
RARα degradation
Ski is overexpressed
N-CoR, HDAC3 degradation
26S Proteasome
HDAC3
N-CoR
N-CoR/SMRT
X
HDAC3
Ski
RXR RARα
Ligand (RA)
RARE
Target Genes OFF
N-CoR/SMRT X
HDAC3
Ski
RXR RARα
Repressive status
RARE
Target Genes OFF
N-CoR/SMRT
X
HDAC3
Ski
RXR RARα
Repressive status
RARE
Target Genes OFF
X
cofactors

Chapter III

Regulation of TBLR1 and Siah2 E3 ligases activity and TBLR1 and Siah2-mediated HDAC3 degradation by the Ski protein

A. Introduction

RA-induced co-repressor complex degradation is important for the subsequent co-activator complex recruitment and target genes transcription initiation (Perissi *et al.*, 2004). Even though we found that Ski might repress RA signaling pathway by stabilizing proteins in the co-repressor complex, the mechanisms involved in this process have not been characterized. In this chapter, I will address potential mechanisms by which Ski can stabilize these proteins in the RA signaling pathway.

It has been reported that retinoic acid receptors are degraded by the 26S proteasome system in response to RA (Boudjelal *et al.*, 2000; Bour *et al.*, 2007; Gianni *et al.*, 2002; Gianni *et al.*, 2003; Kopf *et al.*, 2000; Osburn *et al.*, 2001; Tanaka *et al.*, 2001). Until now, the E3 ligases that are responsible for the retinoic acid receptors recognition and degradation have not been identified. Recently, it has been shown that the HECT domain and Ankyrin repeat containing E3 ubiquitin-protein ligase (HACE1) can interact with the A/B region of RARβ3 (Zhao *et al.*, 2009). Even though HACE1 was initially identified as an E3 ubiquitin ligase (Anglesio *et al.*, 2004), surprisingly, it inhibits RA-induced RARβ3 degradation. The manner of interaction between HACE1 and RARβ3 might block the E3 ubiquitin ligase activity of HACE1 and prevent its function as an E3 ligase for RARβ3. Therefore, the exact E3 ligase for the retinoic acid receptors is still unknown.

Based on our data and data from others (Perissi *et al.*, 2004), we know that HDAC3 also undergoes proteasome degradation upon RA treatment. Two proteins have been indicated to be responsible for HDAC3 degradation. One is TBL1 and the other is Siah2. Siah2 overexpression resulted in HDAC3 degradation, which could be partially

abrogated by the overexpression of TBL1ΔN (a dominant-negative form of TBL1). These data suggest that HDAC3 degradation is TBL1 dependent (Perissi *et al.*, 2004).

TBL1 (transducin beta-like protein 1), an F box/WD-40-containing protein, was first isolated as a novel X-linked gene and related to an X-linked human disorder called Ocular Albinism with late-onset Sensorineural Deafness (OASD) (Bassi *et al.*, 1999). Subsequently, TBL1 was co-purified along with the SMRT complex, which also contained HDAC3 (Guenther *et al.*, 2000). Ebi, the homolog of TBL1 (Dong *et al.*, 1999) in Drosophila, has been reported to be responsible for EGFR-mediated Tramtrack88 degradation, which is a repressor of neuronal differentiation (Boulton *et al.*, 2000; Dong *et al.*, 1999). TBL1 was responsible for N-CoR and HDAC3 degradation by recruiting the ubiquitin-proteasome complex, which was required for nuclear receptor-mediated transcriptional activation (Perissi *et al.*, 2004). Furthermore, TBLR1 (transducin beta-like related protein 1) was also identified as a component of the N-CoR/SMRT complex and shared very high homology with TBL1 (Zhang *et al.*, 2002).

TBL1 and TBLR1 have specificity for nuclear receptor regulation. For example, TBLR1 function is required for most nuclear receptors-mediated transcriptional activation. TR, ER and PPAR additionally require TBL1, whereas RAR-mediated transactivation exclusively relies on TBLR1. In addition to nuclear receptors, TBL1 and TBLR1 are also involved in some other transcription factors-mediated transcription. NF-κB-mediated activation requires both TBL1 and TBLR1, and depletion of either of them leads to the loss of activation of NF-κB target genes in response to TNFα stimulation. However, AP-1-dependent activation only requires TBLR1 (Perissi *et al.*, 2004). Since only TBLR1 is required for RAR-mediated transcriptional activation, TBLR1 is a

candidate that might be responsible for HDAC3 and/or RARα degradation in RA signaling pathway.

The other candidates we are also interested in are Siah proteins. Siah (Seven in Absentia Homolog), the homolog of SINA (Seven in Absentia) in drosophila, are RING finger domain containing proteins and can function as ubiquitin. SINA is required for specification of R7 cell fate in drosophila eyes (Carthew and Rubin, 1990). Several reports indicate that in cooperation with Phyllopod (PHYL), Ebi and UBCD1 (an ubiquitin conjugating enzyme), SINA protein is responsible for ubiquitination and proteasome degradation of the transcriptional co-repressor tramtrack 88 (TTK88). Since TTK88 plays roles in cell fate determination during eye development (Lai *et al.*, 1996; Xiong and Montell, 1993), the physical interaction between SINA and TTK88 and subsequent SINA-mediated TTK88 degradation might be responsible for photoreceptor differentiation (Tang *et al.*, 1997). Two Siah proteins have been identified in human, Siah1 and Siah2. They are also responsible for diverse protein degradation including N-CoR (Zhang *et al.*, 1998), PHD1 and PHD3 (Nakayama *et al.*, 2004), CTBP-interacting protein (Germani *et al.*, 2003), HIPK2 (Winter *et al.*, 2008), FIH (Fukuba *et al.*, 2008), ORF45 protein encoded by Kaposi's sarcoma-associated herpes virus (Abada *et al.*, 2008), and others. Since Siah2 binds to N-CoR and mediates its proteasome degradation, it might be also involved in the HDAC3 and/or RARα proteasome degradation.

In this chapter, we looked at the consequences of expression of TBLR1 and Siah2 on HDAC3 and RAR degradation and determined how co-expression of Ski influenced this degradation. We showed that TBLR1 accelerated HDAC3 proteasome degradation, which could be partially abrogated by the presence of Ski. We also found that Siah1 and

Siah2 expression could accelerate HDAC3 and RARα degradation. In addition, Siah2 was in the same complex with HDAC3, but not with RARα. More interestingly, Ski could stabilize HDAC3 and RARα when it was associated with Siah2, whereas Ski truncation mutants, which could not bind to Siah2, no longer stabilized these two proteins. Furthermore, the Ski protein could stabilize Siah2 expression. All these data indicated both TBLR1 and Siah2 were involved in HDAC3 degradation and Ski stabilized HDAC3 by inhibiting TBLR1 and Siah2 function directly or in indirectly, which might lead to RARα stabilization indirectly.

B. Results

1. TBLR1 targeted HDAC3 for proteasome degradation, which could be partially blocked by the Ski protein.

TBLR1 may be an E3 ligase responsible for HDAC3 degradation, since expression of a dominant negative form of TBL1, the homology of TBLR1 can abolished the degradation of N-CoR and HDAC3 (Perissi *et al.*, 2004). To test the possibility that TBLR1 may affect the stability of HDAC3, we investigated the role of TBLR1 on HDAC3 ubiquitination. By performing His-Ub assay, we found that HDAC3 was polyubiquitinated and the ubiquitinated HDAC3 degraded very fast when TBLR1 was co-expressed. MG132 treatment could recover both the ubiquitinated HDAC3 and non-ubiquitinated HDAC3 (Figure 3.1A). In the presence of TBLR1, not only the ubiquitinated HDAC3 was degraded but also the total un-ubiquitinated HDAC3 in the cell lysate decreased accordingly. These results provided evidence that TBLR1 could mediate HDAC3 for proteasome degradation. We next assessed whether TBLR1 was responsible for RARα degradation, since Ski could stabilize RARα. COS-1 cells were transfected with Flag-RARα alone, or Flag-RARα and HA-TBLR1. As shown in Figure 3.1B, TBLR1 co-expression could not induce the degradation of RARα and MG132 treatment slightly increased RARα levels, which indicated that in contrast with HDAC3, RARα might not be a real substrate for TBLR1. Furthermore, co-transfection of cells with Ski resulted in partial abrogation of TBLR1-induced HDAC3 degradation on the one hand, and on the other hand, TBLR1 co-expression also antagonized the stabilization effect of Ski on HDAC3 (Figure 3.2). These observations suggested that TBLR1-induced

HDAC3 proteasome degradation might be specific, since it could antagonize the HDAC3 stabilizer function of Ski.

2. Siah1/Siah2 interacted with HDAC3 and targeted HDAC3 for proteasome degradation.

To investigate whether Siah proteins can also function as other E3 ligases for HDAC3 degradation, we checked the levels of HDAC3 in the presence of Siah1 and Siah2. We found that expression of both Siah1 and Siah2 could induce HDAC3 degradation, which could be recovered by MG132 treatment (Figure 3.3A&D). Since Siah proteins can be auto-ubiquitinated, they are undetectable due to their rapid turnover. In the presence of MG132, we could detect them easily. The RING finger domain mutant (RM) of Siah proteins was stable due to the defective E2 conjugating enzyme recruitment capability (Figure 3.4C). To further characterize the association between Siah proteins and HDAC3, we took advantage of the RING finger mutant Flag-Siah2 (RM), in which two amino acids H99 and C102 were mutated to A (H99A/C102A), and co-expressed Flag-Siah2 (RM) with Myc-HDAC3 in COS-1 cells. By performing co-immunoprecipitation, we found Siah2 and HDAC3 were in the same complex (Figure 3.4A). In contrast, we could not detect the association between Siah1 and HDAC3 (Figure 3.4D). The protein stability data suggested that both Siah1 and Siah2 could mediate HDAC3 proteasome degradation. Since only Siah2 interacted with HDAC3, we suspected that Siah2 might a real E3 ligase for HDAC3 proteasome degradation. However, we could not exclude the possibility that HDAC3 was also a substrate of Siah1.

3. Siah1/Siah2 could down regulate RARα levels, but they were not in the same complex with RARα.

Similarly, we characterized the effects of Siah proteins on RARα levels. As can be seen in Figure 3.3B, C and E, both Siah1 and Siah2 could accelerate RARα proteasome-dependent degradation. However, we could not detect any interaction between RARα and Siah1 or Siah2 (Figure 3.4B and E) under conditions that allowed co-immunoprecipitation of Siah2 and HDAC3 (Figure 3.4A). Like the effect of TBLR1 on RARα stability, we think that the effects of Siah protein expression on RARα degradation might be indirect.

4. Ski interacted with Siah2 and inhibited the activity of Siah2.

Given that Ski could stabilize HDAC3 and RARα, and Siah2 could induce their degradation via the ubiquitin-proteasome pathway, we next investigated the relationship between Siah protein and Ski. First, we performed co-immunoprecipitation assay to determine whether they could interact with each other. As shown in Figure 3.5A and B, Siah2 and Ski interacted with each other and were both present in the same immunoprecipitation complex. Interestingly, we could not detect a similar interaction between Siah1 and Ski. In order to confirm the interaction between Siah2 and Ski, we further carried out reciprocal co-immunoprecipitation assay. We could also demonstrate the interaction of Siah2 with Ski by immunoprecipitating either Flag-Ski using anti-Ski antibody or Flag-Siah2 (RM) using anti-Flag antibody (Figure 3.5C and D). We also could demonstrate the interaction between wild type Siah2 and Ski by treating cells with MG132 to inhibit Siah2 auto-degradation (Data not shown).

Since the interaction between Siah2 and Ski might help us to understand the stabilization mechanism of Ski expression on HDAC3, we further characterized the binding region on Ski that was responsible for Siah2 interaction. We made N-terminal

and C-terminal Ski truncation mutants T7-Ski (1-491) and Flag-Ski (491-728)). By performing co-immunoprecipitation assay, we found that the N-terminus of Ski was responsible for Siah2 interaction (Figure 3.6). Interestingly, the Ski and N-terminal Ski (1-491) proteins that bound to Siah2 could stabilize HDAC3 and RARα, whereas the C-terminal Ski (491-728) protein that did not associated with Siah2 could not prevent HDAC3 and RARα degradation (Figure 3.7). To characterize this phenomenon further, we made several other Ski truncation mutants and determined both their ability to bind to Siah2 and their ability to stabilize HDAC3 and RARα. We found the stabilization effects of Ski truncation mutants on HDAC3 and RARα directly related to whether they could bind to Siah2 (data not shown). The mutants, which could bind to Siah2 strongly, could stabilize HDAC3 and RARα significantly. However, the mutants that bound to Siah2 weakly or not at all were not strong stabilizers for HDAC3 and RARα, these results were summarized schematically in Figure 3.8.

Since the stabilization effects of Ski related to its Siah2 binding ability, we wondered whether Ski could affect Siah2 function by interacting with Siah2. To determine whether Ski could change Siah2 ubiquitin ligase activity, the effect of Ski on Siah2 level was investigated as a measure of the auto-degradation of Siah2. As shown in 3.9A, normally, the Siah2 protein level was barely detectable due to its potent self-ubiquitination and proteasome degradation. MG132 treatment could block Siah2 degradation and made it detectable. Surprisingly, co-expressed Ski could also increase Siah2 protein level indicating Ski had some effect on Siah2 self-ubiquitination and degradation. Since the self-ubiquitination and degradation of Siah2 is a sign of its ubiquitin ligase activity, Ski might inhibit the activity of Siah2. We further checked the

effects of Ski N-terminal and C-terminal truncation mutants on Siah2 protein level and found, besides wild type Ski, the N-terminal Ski, which could bind Siah2, also stabilized Siah2 even though the stabilization effect was not as strong as wild type Ski (Figure 3.9B). The C-terminal mutant Ski (491-728) could not bind to Siah2 and had no effect on the self-degradation of Siah2. All these data suggested that Ski might increase expression of HDAC3 and/or RARα by interacting with Siah2 and inhibiting Siah2 ubiquitin ligase activity.

C. Discussion

The RA signaling pathway plays roles in a variety of cellular events including vertebrate embryonic development, growth, cell differentiation, apoptosis and homoeostasis (Ertesvag *et al.*, 2009; Kastner *et al.*, 1995; Mark *et al.*, 2006; Pino-Lagos *et al.*, 2008) and deregulation of RA signaling relates to many diseases. RARα and HDAC3 are two important regulators for RA signaling pathway. RARα, a ligand-activated transcription factor, can be a component of the co-repressor complex in the absence of RA and a member of co-activator complex in the presence of RA, which are required for the RA induced transcription activation. HDAC3 is part of the co-repressor complex and has deacetylase activity (Guenther *et al.*, 2000; Li *et al.*, 2000; Wen *et al.*, 2000; Zhang *et al.*, 2002), which can mediate the repressive effect of the co-repressor complex on the RA signaling.

Here, we demonstrated that TBLR1 and Siah proteins could function as E3 ubiquitin ligases and mediate HDAC3 proteasome degradation. The ability of Ski to stabilize HDAC3 is potentially through regulating the function of TBLR1 and Siah2. We found Ski could partially block TBLR1-mediated HDAC3 degradation. More interestingly, we have shown that as a Siah2 interaction partner, Ski might function as an inhibitor of Siah2.

Siah2 can interact with N-CoR and target N-CoR for proteasome degradation (Zhang *et al.*, 1998). Furthermore, Rosenfeld group reported that both Siah1 and Siah2 could induce TBL1-dependent N-CoR degradation because the degradation could be restored by TBL1ΔWD40 (acts as a dominant-negative mutant) (Perissi *et al.*, 2004). They also found similar phenomena in the case of Siah2-induced HDAC3 degradation

(Perissi *et al.*, 2004). TBLR1 is a homolog of TBL1 and required for RARα-mediated transactivation. Since we were interested in the RA signaling pathway, we focused on TBLR1. Our data suggested TBLR1 could accelerate HDAC3 degradation via ubiquitin-proteasome pathway. TBLR1 is an F-box protein, which can be a component of multisubunit E3 ligases and responsible for substrate recognition. But we could not detect the interaction between TBLR1 and HDAC3 (data not shown), which implied that either TBLR1 was not the right E3 ligase for HDAC3 and TBLR1-mediated HDAC3 degradation was non-specific or it needed additional help from other proteins in order to recognize HDAC3 as a substrate. Ebi, an F box protein and homolog of TBL1 in drosophila, can play a role in proteasome degradation of several proteins, but no interactions between Ebi and its substrates have been detected in most cases. In order to associate with its substrates, Ebi needed an additional partner to bridge this interaction (Boulton *et al.*, 2000; Dong *et al.*, 1999; Matsuzawa and Reed, 2001), which might explain why we could not detect the direct interaction between TBLR1 and HDAC3. TBLR1-mediated HDAC3 degradation could be partially abolished by the Ski expression, which was a possible mechanism as to how Ski could stabilize HDAC3 and indicated the down-regulation effects of TBLR1 on HDAC3 might be specific. Since we could not detect interaction between Ski and TBLR1 (data not shown), the antagonistic effect of Ski on TBLR1 could be indirect. TBL1 and TBLR1 can act as recruiters of the ubiquitin-proteasome complex. Ski might interfere with the ubiquitin-proteasome complex recruitment function of TBLR1 since both Ski and TBLR1 are members of N-CoR/SMRT co-repressor complex. Under normal conditions, ligand (RA) treatment induces co-repressor complex dissociation from the nuclear receptor and undergoes

proteasome degradation mediated by TBLR1 and/or another E3 ligase. When Ski is overexpressed, it is in the co-repressor complex. Since Ski can interact with several components in the co-repressor complex, Ski may physically hinder the access of ubiquitin-proteasome complex to TBLR1 and thus block degradation.

As another possible E3 ligase responsible for HDAC3 degradation, we further investigate Siah proteins. The data present here suggested that both Siah1 and Siah2 could mediate HDAC3 degradation, which could be inhibited by MG132. Moreover, we demonstrated that HDAC3 was a novel binding partner for Siah2 by performing co-immunoprecipitation assay, which indicated HDAC3 might be a direct substrate of Siah2. Siah proteins have been shown to act as a monomeric RING finger domain containing E3 ligase for some substrates. For example, Siah seems to bind directly to the substrate DCC (deleted in colorectal cancer) and N-CoR, and mediates the formation of polyubiquitin chain on them. It also has been reported that Sina (a Siah homolog in drosophila), together with phyllopod and the F-box protein Ebi, forms an E3 ubiquitin ligase complex, in which Phyllopod functions as an adaptor responsible for linking Sina to its substrate Tramtrack88 (Li *et al.*, 2002). For Siah-mediated β-catenin proteasome degradation, additional protein partners are utilized. Siah forms a complex with SIP, Skp-1 and Ebi. In this complex, SIP and Skp-1 link Siah to Ebi, and Ebi is responsible for substrate β-catenin recognition (Matsuzawa and Reed, 2001). Even though our data showed Siah2 interacted with and mediated HDAC3 proteasome degradation, we could not exclude the possibility that some endogenous adaptor-like proteins were required for either the interaction between Siah2 and HDAC3 or the subsequent proteasome degradation of HDAC3.

Another Siah interactor we found in this study was the Ski protein. Surprisingly, we found Ski was able to interact with Siah2, but not with Siah1. Siah2 has also been shown to interact with N-CoR and mediated N-CoR degradation, thus we suspected that Siah2, instead of Siah1, was involved in the nuclear receptor signaling pathway. The interaction between Ski and Siah2 was further confirmed by reciprocal co-immunoprecipitation assay. Next we assessed Ski N-terminal and C-terminal truncation mutants and found the N-terminal half of Ski was responsible for Siah2 binding. Furthermore, we found only Ski and Ski truncation mutants that were in the same complex with Siah2 could stabilize HDAC3, which led us to investigate the effects of Ski on Siah2 activity. Our preliminary results indicated that Ski regulated the activity of Siah2. While the expression level of Ski was essentially unaffected by Siah2, Siah2 expression was increased by Ski. This suggested that Ski was a regulator rather than a substrate of Siah2. It is known that Siah2 can be auto-ubiquitinated and turnover very fast, which results in the difficulty to detect the Siah2 protein level, so the increased expression of Siah2 indicates the inhibition of Siah2 ubiquitin ligase activity (Hu and Fearon, 1999; Lorick *et al.*, 1999). At this moment, we have no evidence to explain how Ski can regulate Siah2 activity, but there are several possible mechanisms. First, the interaction between Ski and Siah2 might interfere with the substrate binding activity of Siah2. Until now, most of the ubiquitin ligases function as scaffolds for ubiquitin transfer by interacting with both substrates and ubiquitin-conjugating enzyme. By interacting with Siah2, Ski may occupy the binding site, which is normally for specific substrate binding and thus Siah2 cannot recognize its substrates any more. Second, the Ski binding may block the recruitment of ubiquitin-conjugating enzyme (E2) and the ubiquitin molecules

cannot be transferred to Siah2 substrate. Third, the adapter-like proteins for Siah2 may be important for its function in this system, and the interaction between Ski and Siah2 can inhibit the interaction between Siah2 and adapter-like proteins, thus Siah2 cannot function properly.

As for possible E3 ubiquitin ligases that are responsible for RARα degradation, we found that Siah proteins could mediate RARα degradation, which could be abrogated by MG132. However, unlike HDAC3, RARα was not in the same complex with Siah2. It is possible that some specific adaptor proteins are needed for RARα and Siah2 interaction. Another possibility is that Siah proteins are not E3 ligase for RARα. As we discussed before, the repressive effects of Ski on RA signaling pathway is on the co-repressor/co-activator exchange level, which stops the transcription initiation. Since RARα degradation takes places after the transcription, the stability effects of Ski on RARα might be indirect. Therefore, the Siah proteins-mediated RARα proteasome degradation might also be non-specific.

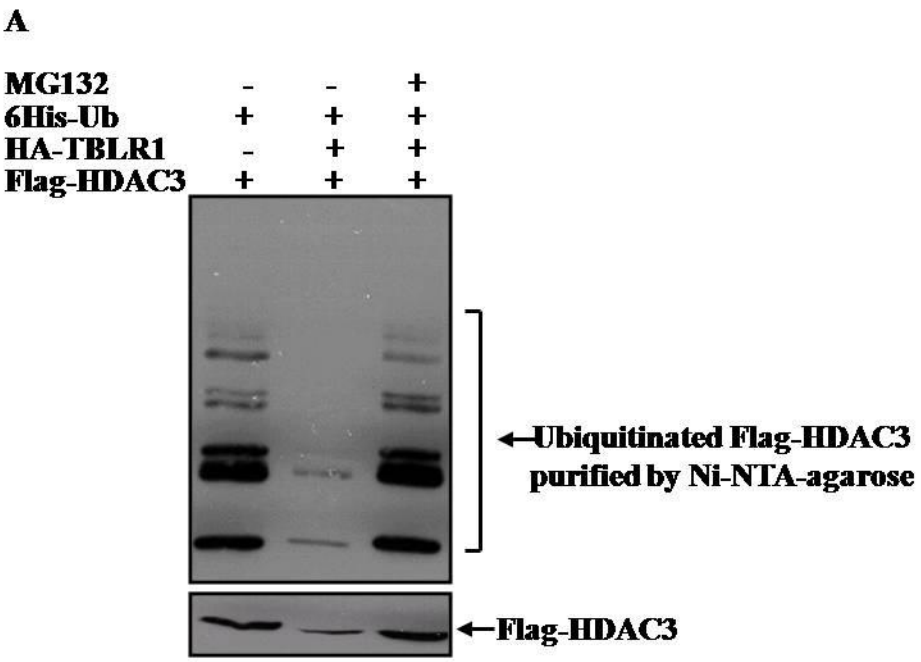
In summary, we identified TBLR1 and Siah2 as two possible E3 ligases that were responsible for HDAC3 proteasome degradation in RA signaling pathway. Potentially Ski could stabilize components in the co-repressor complex by interfering with E3 ligase activity, and thus provided a possible mechanism for the repressive effects of Ski on RA signaling. Regulation of E3 ligase activity, especially Siah2 activity by Ski provides new insights in understanding the role of Ski in transcriptional regulation and the linkage between the ubiquitin-proteasome system and transcriptional regulation. The potential role of Ski as a Siah2 inhibitor also gives us a clue that the function of Ski is involved not only in transcriptional regulation but also in ubiquitin-proteasome system-related cellular

events, which may help explain why the Ski protein plays roles in a variety of biology events and acts as both an oncoprotein and a tumor suppressor.

Figure 3.1 TBLR1 could induce HDAC3 proteasome degradation, but not RARα

A. TBLR1 could accelerate ubiquitinated HDAC3 degradation. QT6 cells were transfected with different combinations of three plasmids: Flag-HDAC3, HA-TBLR1 and 6His-Ub as indicated for 24 hrs, and then were treated with or without MG132 (25μM) for 5 hrs. The ubiquitinated forms of Flag-HDAC3 were purified using Nickel agarose beads, and detected using an anti-Flag antibody by Western blot analysis.

B. COS-1 cells were transfected with Flag-RARα in the presence or absence of HA-TBLR1. After 24 hrs transfection, cells were treated with or without MG132 (25μM) for additional 5 hrs. Cells lysates were analyzed by Western blot using anti-Flag antibody, and β-Actin was used as a loading control.


A
MG132 - - +
6His-Ub + + +
HA-TBLR1 - + +
Flag-HDAC3 + + +
←Ubiquitinated Flag-HDAC3
purified by Ni-NTA-agarose
←Flag-HDAC3

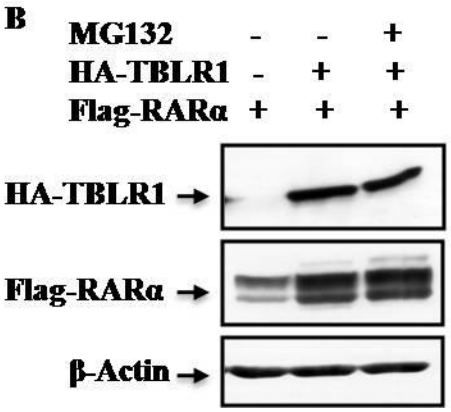

B
MG132 - - +
HA-TBLR1 - + +
Flag-RARα + + +
HA-TBLR1
Flag-RARα
β-Actin

Figure 3.2 TBLR1-induced HDAC3 degradation can be partially inhibited by the Ski protein

COS-1 cells were transfected with 6His-ub and indicated plasmids. After 24 hrs transfection cells lysates were analyzed by Western blot using anti-Flag antibody, and β-Actin was used as a loading control.

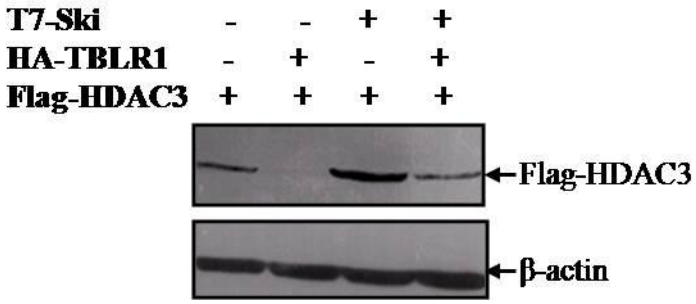

T7-Ski - - + +
HA-TBLR1 - + - +
Flag-HDAC3 + + + +
Flag-HDAC3
β-actin

Figure 3.3 Siah1/Siah2 could mediate HDAC3 and RARα degradation through the ubiquitin-proteasome pathway.

A. To investigate the role of Siah2 in HDAC3 stability, COS-1 cells were transfected with Flag-HDAC3 alone or together with Flag-Siah2. After 24 hrs transfection, cells were treated with MG132 for 5 hrs, and the levels of HDAC3 were detected by Western blot using an anti-Flag antibody.
B. To investigate the role of Siah2 in RARα stability, COS-1 cells were transfected and analyzed as in A.
C. Time dependent Siah2-mediated RARα degradation COS-1 cells were transfected as indicated and were treatment with CHX and RA. Cells lysates were collected at different time points and assayed by Western blot using an anti-Flag antibody to detect Flag-RARα protein levels.
D. Methods were same as A. In this experiment, the effects of Siah1 on HDAC3 levels were checked.
E. Methods were same as B. In this experiment, the effects of Siah1 on RARα levels were checked.

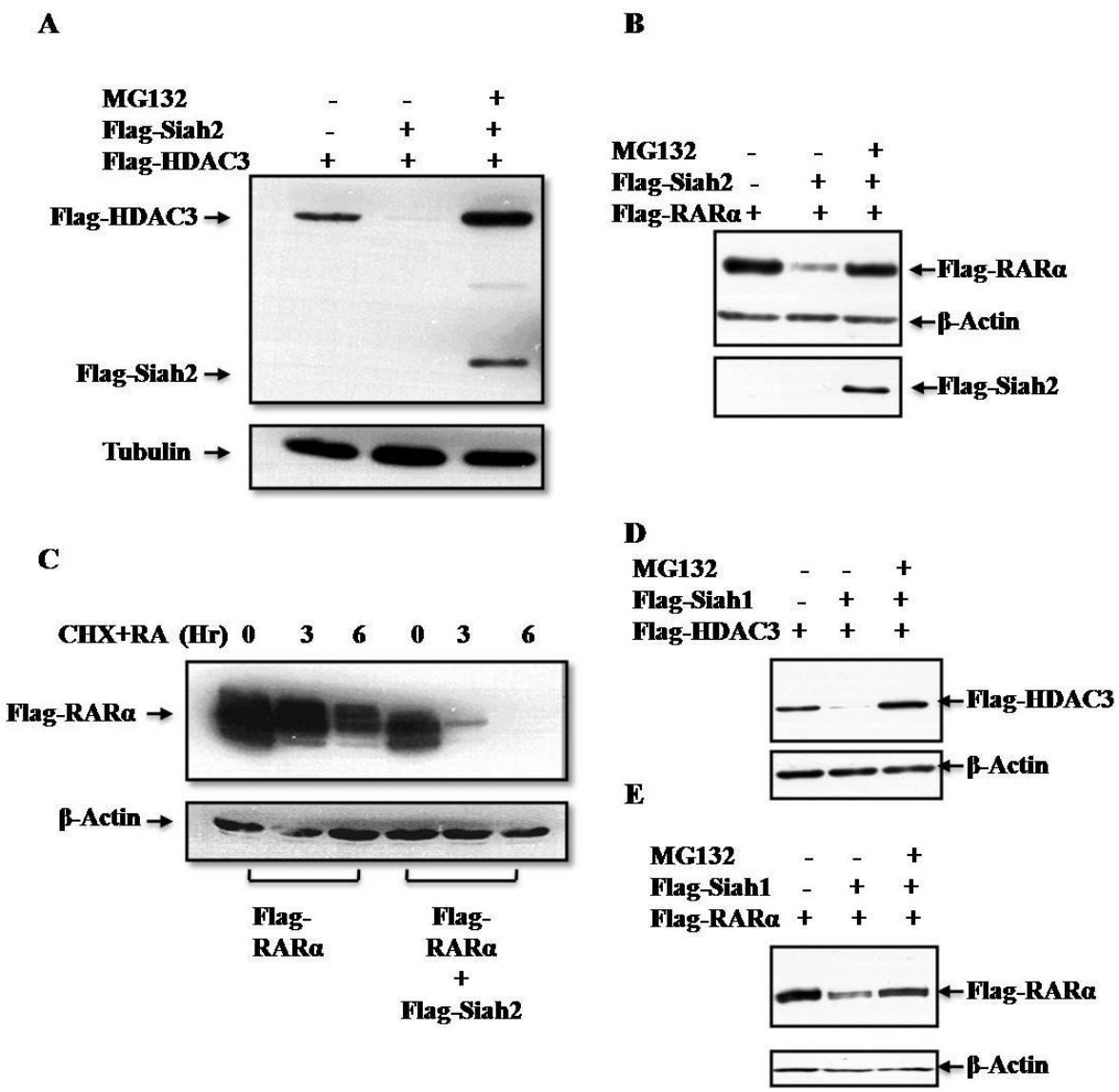

A
MG132 - - +
Flag-Siah2 - + +
Flag-HDAC3 + + +
Flag-HDAC3
Flag-Siah2
Tubulin
B
MG132 - - +
Flag-Siah2 - + +
Flag-RARα + + +
Flag-RARα
β-Actin
Flag-Siah2
C
CHX+RA (Hr) 0 3 6 0 3 6
Flag-RARα
β-Actin
Flag-RARα
Flag-RARα + Flag-Siah2
D
MG132 - - +
Flag-Siah1 - + +
Flag-HDAC3 + + +
Flag-HDAC3
β-Actin
E
MG132 - - +
Flag-Siah1 - + +
Flag-RARα + + +
Flag-RARα
β-Actin

Figure 3.4 Siah2 was in the same complex with HDAC3, but not with RARα. Siah1 was different from Siah2 in that it was not associated with either HDAC3 or RARα.

A. and B. COS-1 cells were transfected with Flag-Siah2 (RM) and Myc-HDAC3 or Myc-RARα. After 24 hrs transfection, cell lysates were collected and used for co-immunoprecipitation assay. Flag-Siah2 (RM) was immunoprecipitated with an anti-Flag antibody, and a mouse normal IgG was used as a control. The immunocomplexes and 10% input were analyzed by Western blot analysis using an anti-Myc antibody (the upper panel) or an anti-Flag antibody (the lower panel).

A. Western blot showed that transfected Siah2 was easily detected when cells were treated with MG132 to block Siah2 proteasome degradation. The RING finger domain mutant Siah2 (RM), with two amino acids H99 and C102 mutated to A, was detectable due to the defective RING finger function.

B. and E. Co-immunoprecipitation assay (same as A and B) showed that Siah1 was not in the same complex with HDAC3 and RARα.

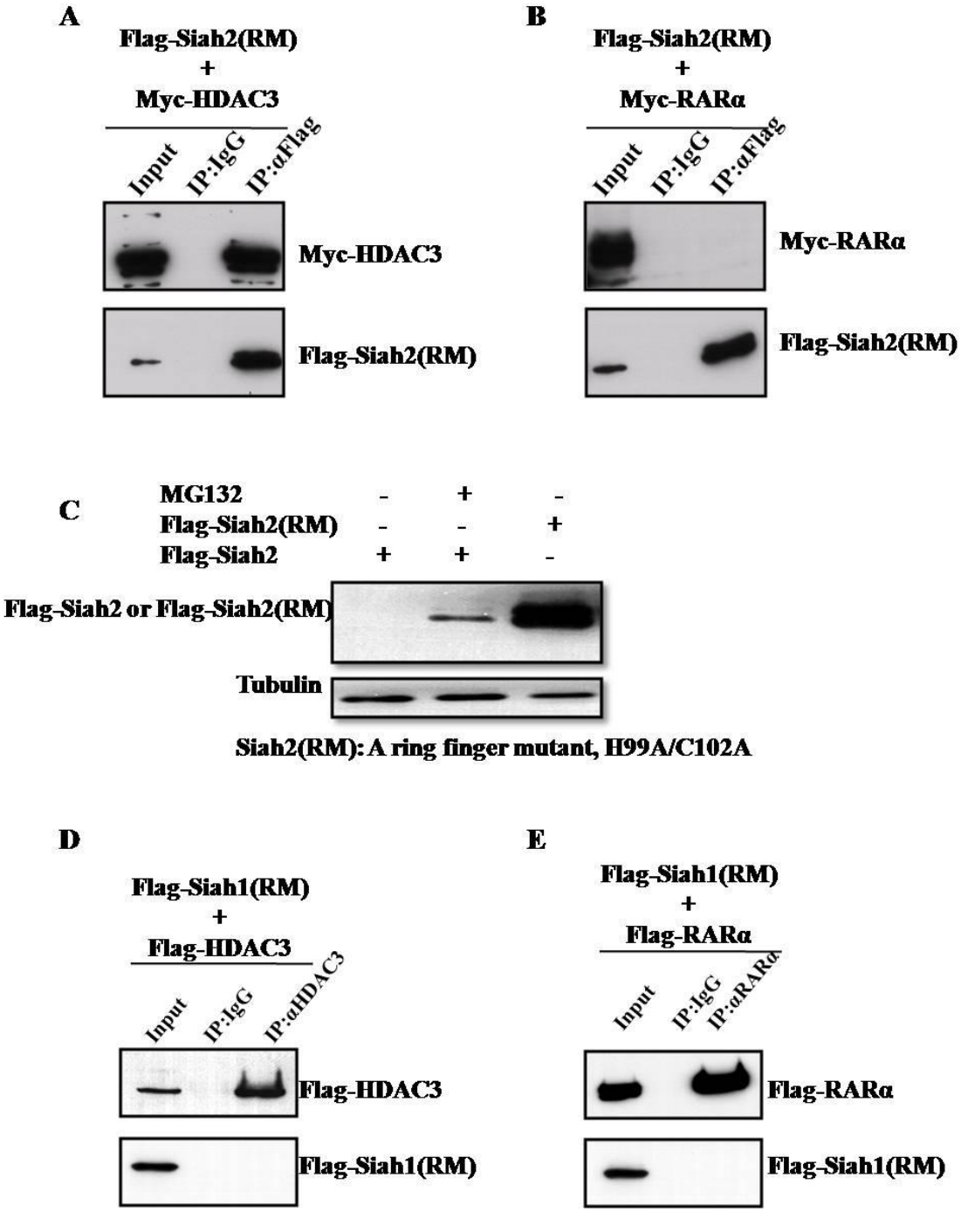

A
Flag-Siah2(RM)
+
Myc-HDAC3
Input
IP:IgG
IP:αFlag
Myc-HDAC3
Flag-Siah2(RM)
B
Flag-Siah2(RM)
+
Myc-RARα
Input
IP:IgG
IP:αFlag
Myc-RARα
Flag-Siah2(RM)
C
MG132 - + -
Flag-Siah2(RM) - - +
Flag-Siah2 + + -
Flag-Siah2 or Flag-Siah2(RM)
Tubulin
Siah2(RM): A ring finger mutant, H99A/C102A
D
Flag-Siah1(RM)
+
Flag-HDAC3
Input
IP:IgG
IP:αHDAC3
Flag-HDAC3
Flag-Siah1(RM)
E
Flag-Siah1(RM)
+
Flag-RARα
Input
IP:IgG
IP:αRARα
Flag-RARα
Flag-Siah1(RM)

Figure 3.5 The Ski protein interacted with Siah2, not with Siah1.

A. and B. Co-immunoprecipitation in COS-1 cells showed the interaction between Ski and Siah2. Flag-Ski and Flag-Siah2 (RM) or Flag-Siah1 (RM) were co-transfected into COS-1 cells. Flag-Ski was immunoprecipitated with an anti-Ski antibody, and a mouse normal IgG was used as a control. The immunocomplexes and 10% input were analyzed by Western blot analysis using an anti-Flag antibody.

C. and D. Reciprocal co-immunoprecipitation assay was performed to confirm the interaction between Ski and Siah2. COS-1 cells were transfected with either Flag-Ski and Flag-Siah2 (RM) or T7-Ski and Flag-Siah2 (RM). Then, we used either and anti-Ski antibody to pull down Ski or an anti-Flag antibody to pull down Siah2 (RM), and checked whether the other protein was in the immunocomplexes by Western blot using proper antibodies.

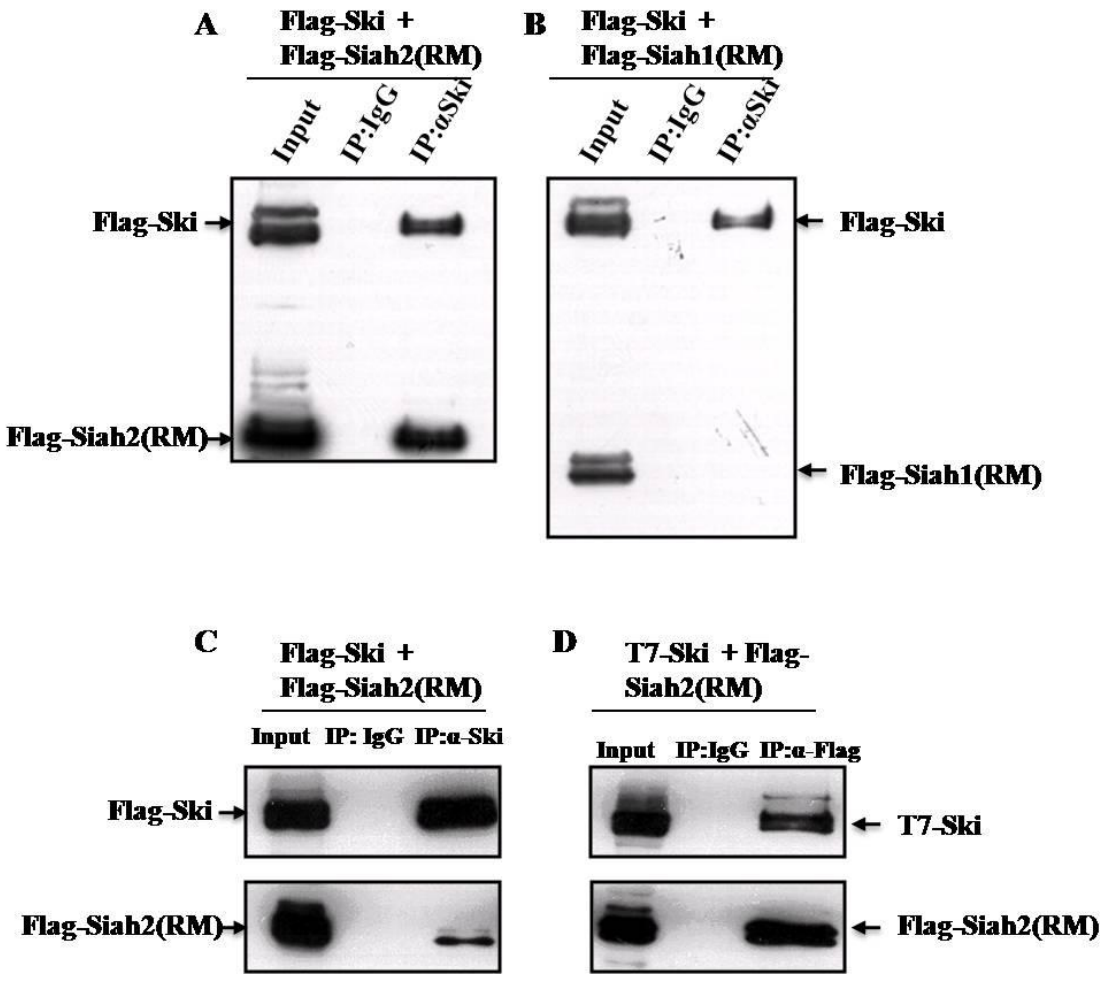
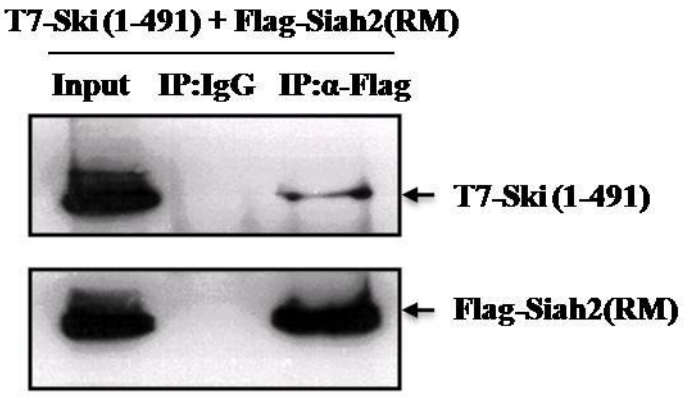

A
Flag-Ski +
Flag-Siah2(RM)
Input
IP:IgG
IP:αSki
Flag-Ski
Flag-Siah2(RM)
B
Flag-Ski +
Flag-Siah1(RM)
Input
IP:IgG
IP:αSki
Flag-Ski
Flag-Siah1(RM)
C
Flag-Ski +
Flag-Siah2(RM)
Input
IP: IgG
IP:α-Ski
Flag-Ski
Flag-Siah2(RM)
D
T7-Ski + Flag-
Siah2(RM)
Input
IP:IgG
IP:α-Flag
T7-Ski
Flag-Siah2(RM)

Figure 3.6 The N-terminal Ski mediated the interaction between Ski and Siah2

A. N-terminal Ski truncation mutant (T7-Ski (1-491)) was made and Siah2 co-immunoprecipitated with T7-Ski (1-491) from co-transfected COS-1 cells. Flag-Siah2 was immunoprecipitated from extracts of COS-1 cells using an anti-Flag antibody. Co-immunoprecipitation was determined by Western blot using an anti-T7 antibody.
B. C-terminal Ski fragment was immunoprecipitated from extracts of co-transfected COS-1 cells using an anti-Ski antibody. The co-immunoprecipitation of Siah2 with C-terminal Ski fragment was determined by using an anti-Flag antibody.

A


T7-Ski (1-491) + Flag-Siah2(RM)
Input
IP:IgG
IP:α-Flag
T7-Ski (1-491)
Flag-Siah2(RM)

B

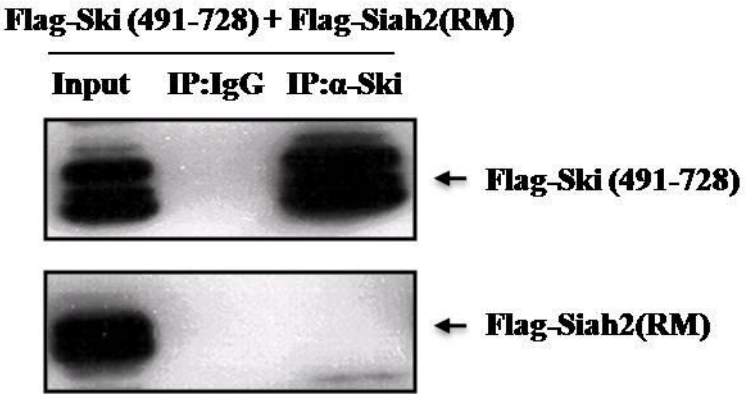

Flag-Ski (491-728) + Flag-Siah2(RM)
Input
IP:IgG
IP:α-Ski
Flag-Ski (491-728)
Flag-Siah2(RM)

Figure 3.7 Ski and N-terminal Ski could stabilize RARα and HDAC3, while the C-terminal Ski could not.

A. COS-1 cells were transfected with Flag-RARα alone or co-transfected with Ski (wild type, N-terminal and C-terminal truncation mutants as indicated) and Flag-RARα. After 24 hrs transfection, cells were treated with CHX and RA for different time as shown in the figure and the Flag-RARα levels were determined and compared by Western blot using an anti-Flag antibody.
B. Same as A. the HDAC3 levels were detected under conditions indicated in the figure.

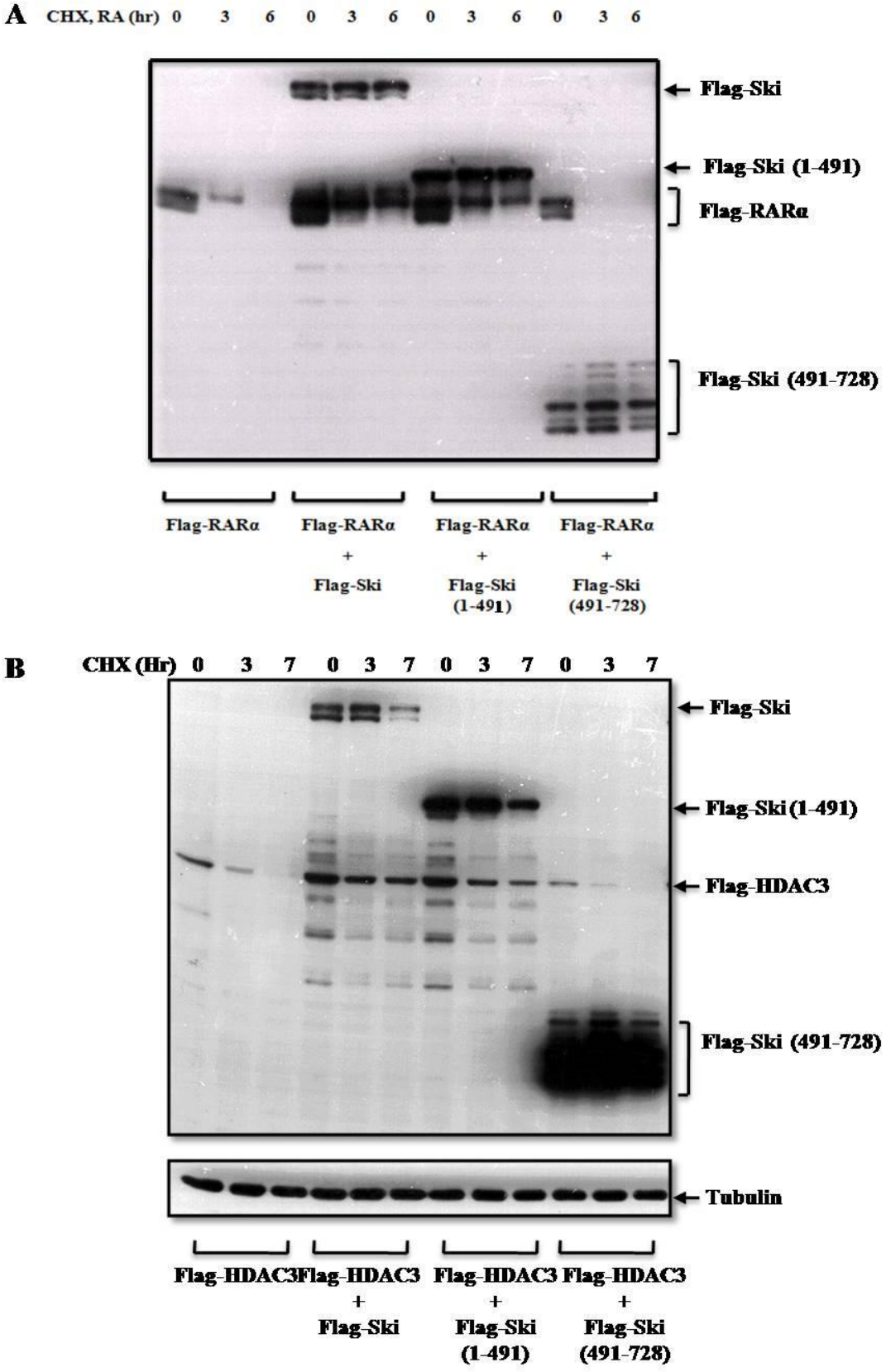

A
CHX, RA (hr) 0 3 6 0 3 6 0 3 6 0 3 6
Flag-Ski
Flag-Ski (1-491)
Flag-RARα
Flag-Ski (491-728)
Flag-RARα
Flag-RARα + Flag-Ski
Flag-RARα + Flag-Ski (1-491)
Flag-RARα + Flag-Ski (491-728)
B
CHX (Hr) 0 3 7 0 3 7 0 3 7 0 3 7
Flag-Ski
Flag-Ski (1-491)
Flag-HDAC3
Flag-Ski (491-728)
Tubulin
Flag-HDAC3
Flag-HDAC3 + Flag-Ski
Flag-HDAC3 + Flag-Ski (1-491)
Flag-HDAC3 + Flag-Ski (491-728)

Figure 3.8 The schematic demonstration of the relationship between Ski and Siah2 interaction and the ability of Ski to stabilize HDAC3 and RARα

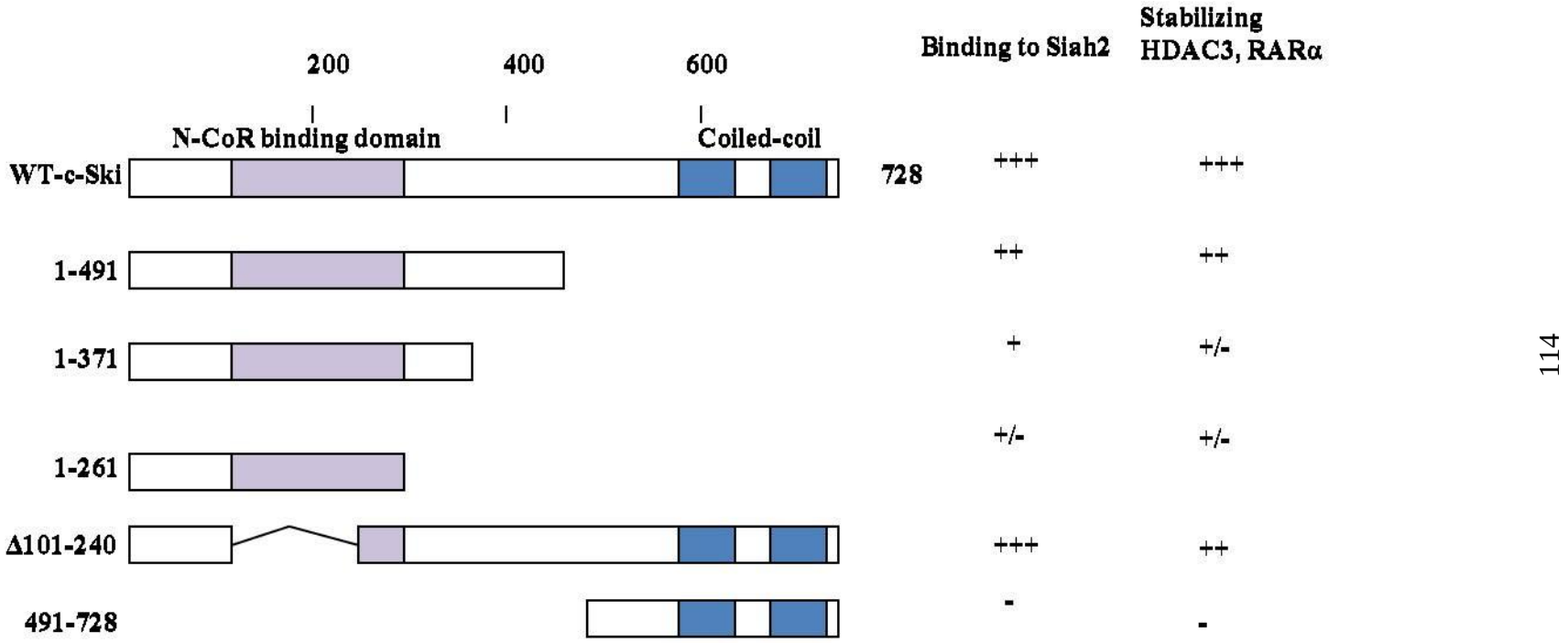
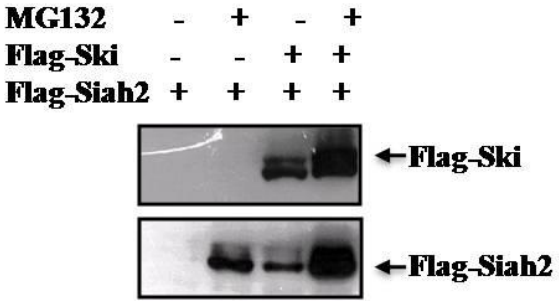

200
400
600
N-CoR binding domain
Coiled-coil
WT-c-Ski
1-491
1-371
1-261
Δ101-240
491-728
Binding to Siah2
Stabilizing HDAC3, RARα
728
+++
+++
++
++
+
+/-
+/-
+/-
+++
++
-
-

Figure 3.9 Ski and N-terminal Ski increased expression of Siah2.

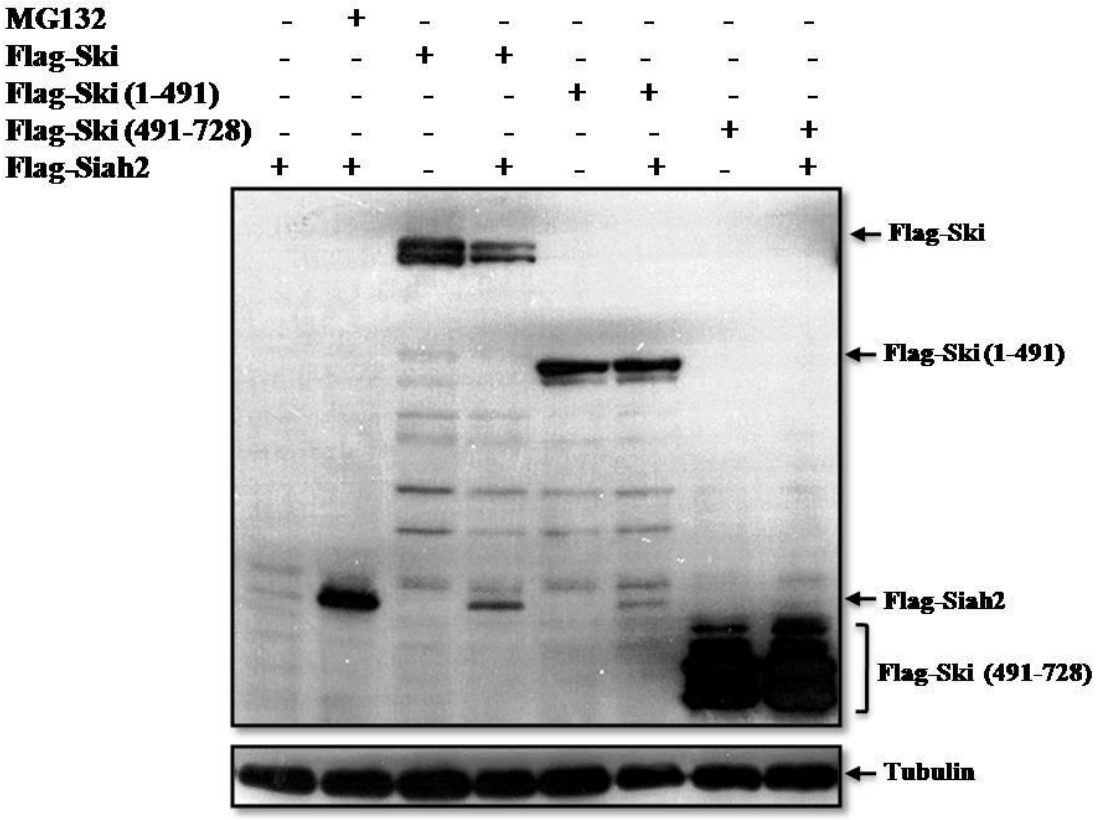
A. COS-1 cells were transfected with Flag-Siah2, or Flag-Siah2 and Flag-Ski together. Where indicated, transfected cells were treated with 25 μM MG132 for 5 hrs before harvesting. Total cell lysates were analyzed by Western blot with an anti-Flag antibody.

B. The wt-Ski, N-terminal and C-terminal Ski truncation mutants were transfected alone or together with Siah2 as described in A. Total cell lysates were analyzed by Western blot with an anti-Flag antibody to determine the Flag-Siah2 levels under various conditions.

A


MG132 - + - +
Flag-Ski - - + +
Flag-Siah2 + + + +
Flag-Ski
Flag-Siah2

B


MG132 - + - - - - - -
Flag-Ski - - + + - - - -
Flag-Ski (1-491) - - - - + + - -
Flag-Ski (491-728) - - - - - - + +
Flag-Siah2 + + - + - + - +
Flag-Ski
Flag-Ski (1-491)
Flag-Siah2
Flag-Ski (491-728)
Tubulin

Chapter IV

Summary, General discussion and Plans

Summary of data and general discussion

ATRA (all-trans retinoic acid) has been clinically used to treat leukemia for more than twenty years by inducing differentiation of the leukemia cells into functional mature cells. Although ATRA can induce complete hematologic remission in most APL patients, ATRA therapy resistant APL patients still exist, and furthermore, ATRA is not effective in other leukemia, such as acute myeloid leukemia (AML) (Breitman *et al.*, 1981; Huang *et al.*, 1988). The Ski oncoprotein can repress retinoic acid pathway and cause the transformation of hematopoietic cells (avian multipotential BM cells and human myeloid cells) (Dahl *et al.*, 1998a; Ritter *et al.*, 2006a). In addition, increased Ski expression can cause highly malignant erythroleukemia (Larsen *et al.*, 1992) and induce immortalization and self-renewal of primary multipotential myeloid progenitor cells from avian bone marrow (Beug *et al.*, 1995). Furthermore, gene expression analysis showed that Ski was up regulated in AML (Ritter *et al.*, 2006b). Even though several studies have demonstrated that the role of Ski's ability to cause leukemia is by blocking or delaying the ability of the cells to differentiate into mature hematopoietic cells, the molecular mechanisms involved in this process were still poorly understood. Therefore, the role(s) and mechanism(s) of the Ski oncoprotein in retinoic acid receptor signaling pathway and in leukemogenesis needed to be further investigated.

The work present here is summarized in Figure 4, which indicates Ski may inhibit RA induced co-repressor/co-activator exchange process by stabilizing components in the co-repressor complex, namely RARα and HDAC3. Furthermore, we identified two E3 ligases responsible for targeting HDAC3 proteasome degradation, TBLR1 and Siah2, and found Ski could not only antagonize TBLR1-mediated HDAC3 degradation but also

inhibit Siah2 E3 ligase activity, which could result in the stabilization of the components of co-repressor complex and subsequent repression of RA signaling pathway.

More and more evidence suggests that the ubiquitin-proteasome system (UPS) closely links to transcription regulation. The UPS functions at several steps in RA signaling pathway. First, the UPS works at the co-repressor/co-activator exchange level, where RA treatment induces the dissociation of co-repressor complex and subsequent degradation of co-repressors via UPS. This degradation is actively mediated by TBLR1, a subunit of E3 ligase complex (Perissi *et al.*, 2004). Second, the UPS is required in transcription activation process. There are several potential explanations for this function of UPS based on experimental evidence. The first evidence is the E3 ligases or proteasome subunits responsible for transcription protein ubiquitination or degradation could be components of the transcription machinery or transcription regulators. The second is ubiquitination of a transcription activation domain is required for transcription activation. In another words, transcription activation domain (TAD)-dependent ubiquitination of transcription factors may not only target them for destruction, but may also be required for their function in transcription activation (Gianni *et al.*, 2002; Salghetti *et al.*, 2001; Verma *et al.*, 2004). Third, the UPS is also responsible for co-activator complex degradation after transcription activation (Lonard *et al.*, 2000). Fourth, the UPS finally mediates RAR/RXR proteasome degradation in order to reset the transcriptional apparatus after each stimulus, so that the previously modified receptors can be replaced with newly synthesized, fully functional receptors (Gianni *et al.*, 2002; Gianni *et al.*, 2003; Tansey, 2001). Our data suggest Ski might repress RA signaling by interfering with UPS at the co-repressor/co-activator exchange level.

First, we demonstrated that the stability of two important components in the co-repressor complex for RA signaling, RARα and HDAC3, were regulated by the Ski protein expression. Ski not only interacted with RARα and HDAC3 but also inhibited the proteasomal degradation of these two proteins. Ski expression slowed down both exogenously expressed and endogenous HDAC3 turnover (Chapter II Figure 2.6C and 2.8). Since Ski can interact with both RARα and components of co-repressor complex, such as N-CoR and HDAC3, Ski might be able to prevent the dissociation of the co-repressor complex from RAR/RXR heterodimer in the presence of RA treatment. We further found that, in addition to this, Ski was also actively involved in inhibiting HDAC3 proteasome degradation by interfering with E3 ligases function. We identified TBLR1 and Siah protein as two E3 ligases that could mediate HDAC3 proteasome degradation. TBLR1, an F box protein, can function as a subunit of E3 ubiquitin ligase complex and is responsible for specific substrate recognition. Even though we found TBLR1 could target HDAC3 proteasome degradation, we could not detect the interaction between them. TBL1, another family member of TBLR1, associates with HDAC3 through SMRT and plays roles in TR signaling pathway (Guenther *et al.*, 2000). Ebi, a homolog of TBL1 in drosophila, was found to associate with its substrates through additional partners in order for it to target the degradation of its substrates (Boulton *et al.*, 2000; Dong *et al.*, 1999; Matsuzawa and Reed, 2001). All this evidence suggests that the TBLR1 and HDAC3 association might also need a bridge. Even though we found Ski could partially abrogate TBLR1 mediated HDAC3 proteasome degradation, we could not detect Ski and TBLR1 interaction either. N-CoR or SMRT might be the possible candidates responsible for

bridging the association between TBLR1 and HDAC3, and even for the interaction between TBLR1 and Ski.

Other E3 ligases potentially responsible for HDAC3 proteasome degradation are Siah1 and Siah2. Surprisingly, we only detected the interaction between Siah2 and HDAC3. Furthermore, Ski could also interact with Siah2, not with Siah1. More importantly, we found only Ski and Ski truncation mutants that were in the same complex with Siah2 had the ability to stabilize RARα and HDAC3. In addition, our data also indicated that Ski regulated the activity of Siah2 since the expression level of Siah2 was stabilized by Ski co-expression. Because Siah2 can be auto-ubiquitinated and degraded through UPS, the increased expression of Siah2 can be indicative of inhibition of Siah2 activity. Recent studies have shown that Siah proteins can be inhibited by several proteins, such as Dab1, Sunphilin-1A, and a fragment derived for a drosophila protein (phyllopod) (Moller *et al.*, 2009; Park *et al.*, 2003; Szargel *et al.*, 2009). The mechanism of how these proteins inhibit Siah function is not clear. The inhibitory effects of a phyllopod fragment on Siah activity was further investigated and it was found this small fragment interferes with Siah-substrate interactions by occupying the binding groove on the Siah protein, which led to stabilization of Siah substrates (Moller *et al.*, 2009). In order to investigate how Ski could inhibit Siah2 activity, we checked whether HDAC3 and Siah2 were in the same complex in the presence of Ski expression by performing co-immunoprecipitation assay. Even though we found both Ski and HDAC3 are present in the Siah2 (RM) immunoprecipitation complex, we did not really know the effect of Ski on HDAC3 and Siah2 interaction since two of Siah2, HDAC3 and Ski could interact with each other (data not shown). Two possibilities can result in immunoprecipitated HDAC3. One is the

immunoprecipitated HDAC3 is from the formation of a tertiary complex, so Ski cannot affect Siah2-substrate binding capability and might function by a different mechanism. The other possibility is that Ski does interfere with the interaction between Siah2 and HDAC3, but HDAC3 is still co-immunoprecipitated since it is associated with Ski.

Siah1 protein seemed also responsible for HDAC3 proteasome degradation even though we did not detect any interaction between Siah1 and HDAC3. Like TBL1 and Ebi, Sina, the drosophila homolog of Siah proteins, have also been shown to need a protein adaptor for substrate recognition (Li *et al.*, 2002; Matsuzawa and Reed, 2001). Based on the above precedent, Siah1 protein might also be an E3 ligase for HDAC3, but it needs adaptor proteins to mediate substrate recognition. Interestingly, both Ebi and Sina have been reported to need adaptor proteins for their substrate recognition and can function as adaptors for each other, which raises the possibility that TBLR1 and Siah protein might function together to target HDAC3 for proteasome degradation in RA signaling pathway. They can act together as an E3 ligase complex and perform different functions, which are necessary for efficient substrate binding and E2 ubiquitin-conjugating enzyme recruitment. However, we cannot exclude the possibility that they can function separately.

The ability of Ski to stabilize HDAC3, a component of the co-repressor complex in RA signaling pathway, by inhibiting E3 ligase activity provides a possible therapeutic target for ATRA resistant leukemia. Ski has been shown to be up regulated two-three fold in AML patients and related to a poor prognosis (Ritter *et al.*, 2006b), and these may lead to ATRA resistant in AML. According to our data, a combination of ATRA and Ski functional inhibition might help to induce the remission of ATRA resistant leukemia cells.

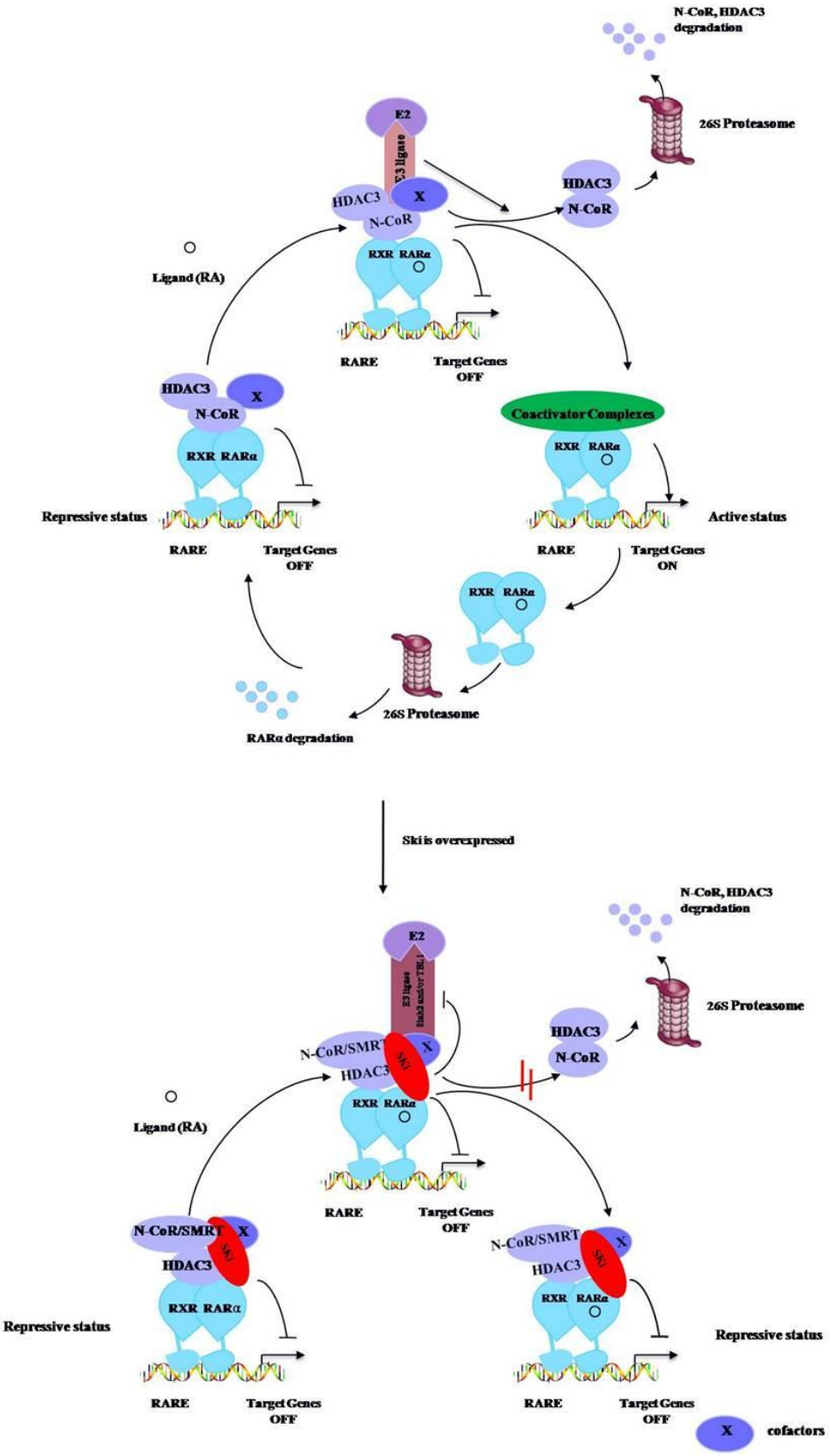
Plans

To fully elucidate the effects of Ski on E3 ubiquitin ligase activity, we have several questions needed to address. First, it will be important to determine if the interaction between Ski and Siah2 is responsible for the inhibition of Siah2 ubiquitin ligase activity. To address this question, isolation of a Ski point mutant that cannot bind to Siah2 could be informative. Siah has been shown to interact with its substrates or other protein partners by recognizing the consensus VXP on their binding proteins (House *et al.*, 2003). There are three VXP containing motifs on the N-terminal Ski protein, it will be interesting to mutate these motifs and analyze the functional differences between wild type Ski and the Ski mutant, such as the binding ability to Siah2, the stability effect on HDAC3, and the inhibitory effect on Siah2 auto-ubiquitination and degradation. Furthermore, siRNA could be designed to target Siah protein to determine whether the stabilization effect of Ski on HDAC3 is dependent on the Siah proteins. We could also take advantage of the Siah2 mutant mice, Siah1a knockout mice and Siah1a/Siah2 double knockout mice, which are potentially available (Frew *et al.*, 2003; Yun *et al.*, 2008), and examine whether the HDAC3 degradation in these Siah-deficient MEF cells are affected. In addition, we can determine whether Ski can still repress RA signaling under the condition of Siah knocked down or mutated.

Another question we should explore is the relationship between TBLR1 and Siah proteins in mediating HDAC3 degradation. Do they use each other as adapters or function independently? If they function corporately, what role do they play respectively? How does Ski link to this process and inhibit their function? It would provide another way to control accurate RA signaling and prevent abnormal signaling seen in many

human diseases if we can understand how TBLR1 and Siah proteins can mediate HDAC3 degradation and how Ski can inhibit Siah2 function.

Figure 4 Model for the mechanism of Ski's repressive effects on RA signaling pathway. See text for details.


N-CoR, HDAC3
degradation
26S Proteasome
E2
E3 ligase
HDAC3
X
N-CoR
HDAC3
N-CoR
RXR RARα
Ligand (RA)
RARE
Target Genes
OFF
HDAC3
X
N-CoR
RXR RARα
Repressive status
RARE
Target Genes
OFF
Coactivator Complexes
RXR RARα
Active status
RARE
Target Genes
ON
RXR RARα
26S Proteasome
RARα degradation
Ski is overexpressed
N-CoR, HDAC3
degradation
E2
E3 ligase
Hdm2 and/or TRIM37
26S Proteasome
N-CoR/SMRT
X
HDAC3
SKI
HDAC3
N-CoR
RXR RARα
Ligand (RA)
RARE
Target Genes
OFF
N-CoR/SMRT
X
HDAC3
SKI
RXR RARα
Repressive status
RARE
Target Genes
OFF
N-CoR/SMRT
X
HDAC3
SKI
RXR RARα
Repressive status
RARE
Target Genes
OFF
X
cofactors

Chapter V

Materials and Methods

Cell Culture, Reagents, and Transfection

COS-1, HeLa, QT6, MEF Ski$^{-/-}$ (a gift from Dr. C. Colmenares, Lerner Research Institute, Ohio, USA), and MG63 cells were maintained in Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml). For QT6 cells, medium was additionally supplemented with 1% chicken serum (Sigma, St Louis, MO, USA). Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Expression plasmids were introduced into the cells using FuGENE 6 (Roche Applied Science, Indianapolis, IN, USA) or TransIT-LT1 (Mirus Bio LLC, Madison, WI, USA) as described by the manufacturer. Briefly, the recommended amount of FuGENE 6 or TransIT-LT1 was diluted into 150 μl DMEM medium and incubated for 5 minutes at room temperature. Then DNA or different DNA combinations were added into the medium and the mixture was incubated for another 15-20 minutes prior to addition to the cells.

Plasmid Constructs

T7-wt-cSki, Flag-RARα, Myc-RARα, RXR, Myc-HDAC3, 6His-Ub and Flag-N-CoR-C were described previously (Marcelain and Hayman, 2005; Ritter *et al.*, 2006a; Ueki and Hayman, 2003b; Ueki *et al.*, 2008). Flag-HDAC3 was constructed by cloning mouse HDAC3 cDNA into the pCMV-Tag2B vector (Stratagene, La Jolla, CA, USA). GFP-Ski was made by cloning hSki cDNA, which was obtained from pSP65-hSki plasmid, into pEGFP-C1 vector (Dr. K. Marcelain made this plasmid). Flag-Ski was constructed by inserting human c-Ski fragment into BamHI site of pCMV-Tag2C vector (Stratagene). Flag-Ski point mutant, L127P, was generated by site-directed mutagenesis

PCR using QuikChange (Stratagene). The PCR reaction was performed on a DNA thermal cycler (Perkin Elmer, Shelton, CT, USA) and the condition of reaction was as follows: 96℃, 1 min for 1 cycle; 96℃ 50 sec, 60℃ 50 sec, 68℃ 6min 48 sec for 18 cycles; 68℃ 7 min and 4℃ for storage. T7-Ski truncation mutant Ski (1-261), Ski (1-371), and Ski (Δ101-240) was generated by inserting PCR-generated human Ski cDNA fragments into BglII and SalI sites of pCMV-T7 vector. The primers used for generation of 1-261 fragment were: 5'- TTTTAGATCTATGGAGGCGGCGGCAGG-3' and 5'-TTTTCTCGAGCACCACGAACTTGTGC-3'. The primers used for generation of 1-371 fragment were: 5'-TTTTAGATCTATGGAGGCGGCGGCAGG-3' and 5'-TTTTCTCGAGGTGAACACAGCCCAGG-3'. The primers used for generation of 1-100 fragment were 5'-TTTTAGATCTATGGAGGCGGCGGCAGG-3' and 5'-CGGGATCCGCGCTCGGTGGAGCGG-3'.The primers used for generation of 241-728 fragment were 5'-CGGGATCCAGCGCCGCCTGCATCCAGTGCC-3' and 5'-TTTTCTCGAGCTACGGCTCCAGCTCCGC-3'. Fragments 1-100 and 241-728 were ligated together and inserted into the BglII and SalI sites of pCMV-T7 vector. Flag-Ski (1-491) was made by cutting Flag-Ski plasmid at EcoR1 sites (one EcoR1 site is at amino acid 491 on the Ski protein, the other EcoR1 site is in pCMV Tag2C vector) and then purifying the large fragment and ligated with pCMV Tag2C vector together. Flag-Ski (491-728) was made by cutting Flag-Ski plasmid at EcoR1 sites (one ECoR1 site is at amino acid 491 on Ski protein, the other one ECoR1 site is on pCMV Tag2C vector), and then inserting the fragment into Tag2B vector digested with EcoR1. T7-Ski (1-491) was made by similar method to Flag-Ski (1-491). Ski-specific small hairpin RNA (target sequence: gtactcggcccagatcgaa) was cloned into LMP retroviral vector (a gift from Dr.

S.W. Lowe, Cold Spring Harbor Laboratory, NY, USA). Inducible hSki expression plasmid was generated by inserting hSki fragment into pRVYtet vector which was described in (Sweasy *et al.*, 2005). Using this system, we could turn on Ski expression by removing Doxycycline. All mutagenesis and cloning procedures were verified by DNA sequencing. T7-Ski (1-491), Flag-Ski (1-491), Flag-Ski (491-728), LMP-shRNA against Ski and pRVYtet-Ski were generated by Dr. N. Ueki. Flag-Siah2 and Flag-Siah2 (RM), which is a ring finger mutant with two amino acids mutated (H99A/C102A) were gifts from Dr. D. Bar-Sagi, New York University. HA-TBLR1 plasmid was a gift from Dr. J.M. Wong, Baylor College of Medicine.

Whole cell lysates preparation

Cells were washed with PBS twice and scraped into a 1ml Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 10% glycerol) containing protease inhibitor phenylmethylsulfonylfluoride (PMSF) and phosphatase inhibitors NaF(10mM) and sodium vanadate (1mM). Then the collected cells were sonicated briefly on Branson sonifier 450 (VWR scientific, West Chester, PA, USA) at 2.5 output. After sonication, the cell lysates were clarified at 13,000rpm for 10 min at 4℃. The supernatant was taken as whole cell lysate and stored at -20℃ for future use.

Protein stability assay

COS-1 cells were transiently transfected with plasmids as indicated using FuGENE 6 (Roche Applied Science) or TransIT-LT1 (Mirus Bio LLC) transfection reagents as described by the manufacturers. 24h after transfection, culture medium was added cycloheximide (CHX, 50μl) (Sigma) and/or retinoic acid (RA, 1μM) (Sigma).

Cells were lysed at indicated time after addition of CHX and/or RA. Lysates were analyzed by Western blot.

Co-immunoprecipitation assay and Western blot analysis

Cells were lysed in Nonidet P-40 buffer. After sonication and clarification, extracts were precleared with protein G-Sepharose or protein A-sepharose (GE Healthcare, Piscataway, NJ, USA) for 1 h at 4℃ on a rotating wheel. Precleared extracts were incubated with appropriate antibodies or normal mouse IgG or normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunocomplexes were recovered using protein G-Sepharose or protein A-sepharose (GE Healthcare) overnight at 4℃ on a rotating wheel, followed by washing four times with Nonidet P-40 buffer. Recovered proteins were separated by SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, NH, USA). Antibodies used for immunoprecipitation assay and Western blot analysis were anti-Myc (9E10, Santa Cruz Biotechnology), anti-Flag mouse monoclonal antibody (Sigma), anti-T7 mouse monoclonal antibodies (Novagen, Madison, WI, USA), anti-Ski (H-329, Santa Cruz Biotecnology), anti-HDAC3 (3G6, Upstate, Charlottesville, VA, USA), anti-Ski (G8, Cascade Bioscience, Winchester, MA, USA), anti-RARα (Santa Cruz Biotechnology), anti-β-Actin (Sigma), and anti-α-Tubulin (Sigma). Proteins were detected with the appropriate secondary antibodies (GE Healthcare) by chemiluminescence (Perkin Elmer).

Luciferase reporter assays

QT6 cells in 24-well plate were transfected with the luciferase reporter construct CRBPII-Luc (50 ng/well) (a gift from Dr. Vimla Band, Tufts University School of Medicine, Boston, MA, USA) (Zeng *et al.*, 2002) or construct TK-Luc (50 ng/well), and

β-galactosidase (TK-βGal, 50 ng/well), plus effector plasmids (100 ng/well). We equalized total amount of transfected DNA with empty vector DNA (pEGFP-C1). Luciferase activities were measured 24 hrs after transfection and normalized to the β-galactosidase activities according to the manufacturer's instructions (Promega, Madison, WI, USA). The results were mean values and S.D. from three independent experiments. For ligand stimulation, we treated cells with retinoic acid (1 μM) or appropriate solvent 24 hrs after transfection.

Indirect immunofluorescence and microscopy

Indirect immunofluorescence and microscopy were carried out as described previously (Marcelain and Hayman, 2005). HeLa cells were transfected with GFP- Ski and Myc-RARα in the presence or absence of RA. Then, they were fixed with 3% paraformaldehyde for 15 min and permeabilized with PBS-0.5% Triton X-100 for 10 min. After permeabilization, cells were blocked with 3% BSA for 45 min. Anti-RARα was diluted 1:200 in PBS-1% BSA and used to detect RARα, Alexa Fluor 546 goat anti-rabbit (Invitrogen) was used as secondary antibody. Hoechst was used for DNA staining. Cells were visualized with an Axiovert 200M (Zeiss, Thornwood, NY, USA) using a 63X oil DIC lens and the images were analyzed using the Axiovision software (Zeiss).

Proteasome inhibition and in vivo ubiquitination assays

The proteasome inhibitor MG132 (Sigma) was used at a concentration of 25 μM or 5μM for the time indicated in the legend of figures. Ubiquitinated intermediates in human cells were detected using 6His-tagged-ubiquitin (6His-Ub) as described previously (Marcelain and Hayman, 2005). QT6 cells were transfected with Flag-RARα (1 μg), 6His-Ub (1 μg) either in the absence or in the presence of plasmid T7-Ski. After

24 hrs transfection, cells were treated with RA for 2 hrs and harvested. The ubiquitinated proteins were recovered by nickel-affinity chromatography and analyzed by Western blot analysis

Retrovirus-mediated Gene Transfer

The amphotropic retroviral packaging cells Phoenix A were plated at a density of 2 x 10^6 cells/60-mm dish and transfected with each plasmid DNA. Virus-producing cells were grown for 48 hrs to confluence prior to harvesting the viral supernatant. Cells were infected with the supernatant supplemented with Polybrene (10 μg/ml) and incubated for 6 hrs. We cultured the infected cells for 48 hrs in fresh medium and then selected for 1 week in the presence of appropriate antibiotics. Briefly, MEF $Ski^{-/-}$ cells, infected with pRVYtet-hSki were cultured for 48 hrs in fresh medium and selected for 1 week in the presence of Hygromycin (50 μg/ml) (Invitrogen). Then individual clones were established. MG63 osteosarcoma cells, infected with LMP-Ski-shRNA, were cultured for 48 hrs in fresh medium and then selected in the presence of Puromycin (2 μg/ml) (Sigma) for 1 week.

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